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Award Number: DAMD17-97-1-7172

TITLE: Regulation of Breast Tumor Angiogenesis by Interactive FGFR-Notch Signaling

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REPORT DATE: August 2000

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	August 2000	Final (1 Jul 97 - 1 Jul 00)

4. TITLE AND SUBTITLE
Regulation of Breast Tumor Angiogenesis by Interactive FGFR-Notch Signaling

5. FUNDING NUMBERS
DAMD17-97-1-7172

6. AUTHOR(S)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
This report contains colored photos

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT ***(Maximum 200 Words)***

Jagged-Notch interactions regulate a transmembrane ligand-receptor signaling pathway involved in the regulation of cell fate determination as well as myoblast and endothelial cell differentiation. To further examine the role of the transmembrane ligand, Jagged-1, in the regulation of cell differentiation, we stably transfected NIH 3T3 cells with a truncated form of Jagged(J)-1, which results in the secretion of a soluble(s) form of the protein. Comparison of gene expression by serial analysis demonstrated that among the 227 transcripts differentially regulated in the sJ-1 transfectants, the expression of the pro-α-2(I) collagen transcript and pro-α(I) collagen translation product was predominantly repressed in sJ-1 transfectants. When plated on extracellular matrices, sJ-1 transfectants formed prominent chord-like structures on type I collagen but not on fibrin, fibronectin, or vitronectin. While the sJ-1 transfectants exhibited growth kinetics similar to control cells and were unable to grow in soft agar, the cells were less sensitive to contact inhibition of growth in vitro and sJ-1 allografts formed tissue masses in nude mice after a prolonged latency period and exhibited an abundance of host-derived microvascular endothelial cells. These data suggest that J-1 may be able to modulate, in ability to promote the development of chord-like structures.

14. SUBJECT TERMS
Breast Cancer

15. NUMBER OF PAGES
70

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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David M. Mann 11/1/00

PI - Signature Date

Table of Contents

Introduction:

The object of this proposal was to assess the role of Jagged1 as a modifier of angiogenesis, an event important for the development of breast tumor growth and metastasis.

Body:

The following have been accomplished:

(i) Study of biochemical and cytological effects of the expression of soluble Jagged1.

(ii) Investigation of cooperation between FGF1 and Jagged1 signaling pathways.

(iii) Examination of the expression of Notch receptors and ligands in the course of endothelial cell migration following balloon angioplasty *in vivo*.

Key Research Accomplishments:

(i) Soluble Jagged1 is an angiogenesis factor *in vivo*

(ii) Soluble Jagged1 expression results in a specific phenotype which includes:

- (a) the formation of collagen-dependent cords, *in vitro*
- (b) an attenuation of collagen type I mRNA and protein expression
- (c) an attenuation of the F-actin cytoskeleton, focal adhesion sites, and cell migration
- (d) the activation of the Src signaling pathway.

(iii) Most of the characteristics of the Soluble Jagged1-induced phenotype with the exception of decreased migration are Src-dependent and have been shown to be abrogated by the expression of a dominant negative Src construct.

(iv) Unlike control cells, soluble Jagged1 transfectants fail to sustain the activation of MAP kinases upon the stimulation with FGF1 and exhibit an FGF1-dependent transformed phenotype *in vitro*.

(v) Jagged1 and Jagged2 but not Delta1 are strongly upregulated in the regenerating endothelium in vivo while Notch expression (1 through 4) is slightly increased in both endothelial and smooth muscle cells in injured vessels.

Reportable Outcomes:

1. Wong, M.K.K., Prudovsky, I., Vary, C., Booth, C., Liaw, L., Mousa, S., Small, D., Maciag, T. A Non-Transmembrane Form of Jagged-1 Regulates the Formation of Matrix-Dependent Chord-like Structures (2000) Biochem. And Biophys. Research Commun. 268:853-859

2. Small, D., Kovalenko, D., Marek, D., Liaw, L., Landriscina, M., Di Serio, C., Prudovsky, I., Maciag, T. Src-Dependent Chord Formation Mediated by Jagged 1 Is Independent of Cell Migration (2001) J. Biol. Chem. submitted.

3. Lindner, V., Booth, C., Prudovsky, I., Small, D., Maciag, T., Liaw, L. Members of the Jagged/Notch Gene Families Are Expressed in Injured Arteries and Regulate Cell Phenotype via Alterations in Cell-matrix and Cell-cell Interaction, (2001) Am. J. Pathol. Submitted.

Conclusions:

(i) FGF/FGFR, Jagged/Notch and Src signaling pathways cooperate in the processes of angiogenesis and endothelial cell differentiation.

(ii) Jagged/Notch signaling appears to participate in the regeneration of damaged blood vessels.

(iii) Jagged1 expression may cooperate with members of FGF gene family in the promotion of angiogenesis during the development of human breast tumors.

References:

See Appendix

Biochemical and Biophysical Research Communications **268,** 853–859 (2000)
doi:10.1006/bbrc.2000.2173, available online at http://www.idealibrary.com on IDEAL®

A Non-Transmembrane Form of Jagged-1 Regulates the Formation of Matrix-Dependent Chord-like Structures

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Received November 26, 1999

Jagged-Notch interactions regulate a transmembrane ligand-receptor signaling pathway involved in the regulation of cell fate determination as well as myoblast and endothelial cell differentiation. To further examine the role of the transmembrane ligand, Jagged-1, in the regulation of cell differentiation, we stably transfected NIH 3T3 cells with a truncated form of Jagged(J)-1, which results in the secretion of a soluble(s) form of the protein. Comparison of gene expression by serial analysis demonstrated that among the 227 transcripts differentially regulated in the sJ-1 transfectants, the expression of the pro-α-2(I) collagen transcript and pro-α-1(I) collagen translation product was predominantly repressed in sJ-1 transfectants. When plated on extracellular matrices, sJ-1 transfectants formed prominent chord-like structures on type I collagen but not on fibrin, fibronectin, or vitronectin. While the sJ-1 transfectants exhibited growth kinetics similar to control cells and were unable to grow in soft agar, the cells were less sensitive to contact inhibition of growth *in vitro* and sJ-1 allografts formed tissue masses in nude mice after a prolonged latency period and exhibited an abundance of host-derived microvascular endothelial cells. These data suggest that J-1 may be able to modulate, in a matrix-dependent manner, the organization of cell to cell interactions including its ability to promote the development of chord-like structures. © 2000 Academic Press

Angiogenesis is an integral part of physiologic and pathologic processes such as embryonic development, wound repair, solid tumor growth, and chronic inflammation, and involves the ability of the endothelial cell to coordinate migration, proliferation, and differentiation pathways to form new vascular structures (1, 2). While the ability of the angiogenic growth factors such as vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF) to initiate endothelial cell migration and growth are well described (3, 4), the identification of factors involved in the regulation of the tubular, chord-like vascular phenotype has been difficult to access. We have previously reported that the transmembrane protein, J-1, a ligand for its transmembrane receptor Notch (5), is involved in the regulation of human endothelial cell differentiation *in vitro* (1). Jagged-Notch interactions mediate an evolutionarily conserved intercellular signaling pathway responsible for the regulation of developmental cell fate decisions *in vivo* (6) and cellular differentiation *in vitro* (7, 8). During the cloning of the human J-1 gene, two cDNA clones were isolated which contained identical deletions resulting in the insertion of 15 novel amino acids followed by a premature termination of the J-1 sequence prior to the domain encoding the transmembrane and intracellular sequences (1). Since this truncated J-1 cDNA contained the J-1 signal peptide sequence, we anticipated that cells transfected with this construct would secrete the truncated ectodomain of J-1 as a soluble(s) and extracellular form of the J-1 protein and this would eliminate the transmembrane constraints imposed upon the J-1 ligand to signal by an intercellular pathway. We report that human sJ-1 is able to influence the formation of a matrix-dependent chord-like phenotype *in vitro.*

Abbreviations used: fetal bovine serum, FBS; fibroblast growth factor, FGF; Jagged, J; open-reading frame, ORF; phosphate-buffered saline, PBS; polymerase chain reaction, PCR; serial analysis of gene expression, SAGE; soluble Jagged, sJ; vascular endothelial growth factor, VEGF.

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MATERIALS AND METHODS

Soluble jagged-1 plasmid construction. The soluble myc epitope-tagged J-1 expression vector was generated using two separate sequential polymerase chain reaction (PCR) protocols. Overhang PCR was used to place a consensus Kozak sequence 5′ to the J-1 open-reading

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frame (ORF), and to truncate J-1 immediately 5′ to the transmembrane domain. This construct was assembled by ligating the PCR-modified 5′ and 3′ amplicon into the shuttle plasmid, MW27, which consists of the full-length Jagged-1 cDNA in pBlueScript and was subcloned into the eukaryotic expression vector pMexneo (9) using the newly engineered 5′ EcoR1 and 3′ Xho 1 sites to produce the final product. The forward primer used for the 5′ modifications was 5′-GACTATGCGAATTCG-GATCCGTCGACGCCACCATGGGTTCCCCACGGACACGCG and reverse primer was 5′-CAAGTTCCCCCGTTGAGACA, where the Kozak sequence is underlined. The forward primer used for the 3′ modification was 5′-ATGGACAAACACCAGCAGAA and reverse primer was 5′-TAGTGCTCGAGCTATTACAAGTCTTCTTCAGAAATAAGCT-TTTGTTCATCTGTTCTGTTCTTCAG, where the myc epitope is underlined. The template used for PCR was the complete human J-1 ORF originally obtained from Dr. G. Gray (Yale University). Reactions were performed using Vent polymerase (New England Biolabs) in 1× Vent buffer as recommended by the manufacturer. The thermal cycling parameters consisted of 94°C (1 min) followed by 35 cycles at 94°C (30 sec), 62°C (30 sec), 72°C (30 sec) followed by a 10 min hold at 72°C before termination at 4°C. The 5′ PCR-modified product was digested with EcoRI and BglII, electrophoretically resolved on a 1% (w/v) agarose gel, electroeluted, and ligated with a similarly digested MW27 to create MW13 using standard protocols (10). The modified 3′ PCR-amplified product was similarly processed except that restriction digestion utilized XhoI and AccI. The ligation was performed with a similarly digested MW13 to yield MW32. This 5′-Kozak-truncated J-1 3′-myc-tagged pBlueScript construct was digested with EcoRI and XhoI and ligated into pMexneo. All restriction enzymes and buffers were obtained from New England BioLabs and two sJ-1 transfectant clones, 38-1 and 38-4, and one insert-less vector transfectant clone were used for experimentation.

Cell transfection, immunoprecipitation, immunoblot analysis, and matrix preparation. NIH 3T3 cells were transfected with MW38 using the calcium-phosphate kit (Stratagene) and G418 (Gibco/BRL) selection. Stable sJ-1 transfectants were grown and maintained in DMEM (GIBCO/BRL) supplemented with 400 μg/ml G418 and 10% (v/v) fetal bovine serum (FBS) (HyClone, Logan, UT). G418-resistant cells were grown to confluency in DMEM containing 10% (v/v) FBS, the cells washed twice in phosphate-buffered saline (PBS) and incubated with labeling media consisting of cys- and met-free DMEM supplemented with 1× Nutriderma (Gibco/BRL) and 0.4 μCurie/ml of [^{35}S]-met/cys mixture (DuPont-New England Nuclear). After 4 h, the labeling medium was removed, the cells washed once with ice cold PBS, scraped into 1.0 ml of PBS and pelleted. The cell pellets were resuspended in RIPA lysis buffer containing 1 mM PMSF, 10 μg/ml aprotinin and 1 μg/ml leupeptin (Sigma), clarified by centrifugation (13,000 × g for 10 min) and incubated with 30 μl of Protein-A Sepharose (Pharmacia) which had been complexed with 9E10 monoclonal antibodies to the myc epitope (Oncogene). The immunoprecipitates were washed four times with RIPA buffer, dissolved in 50 μl of 2× SDS sample buffer, and resolved in 8% (v/v) SDS-PAGE as described (11). To assess the secretion of sJ-1, the conditioned medium (1 ml) from the [^{35}S]-met/cys-labeled cells were collected in 1 mM PMSF and 10 μg/ml aprotinin then incubated with 50 μl of Protein A-Sepharose and treated as outlined above except that the immunoprecipitates were washed six times prior to being dissolved in SDS sample buffer.

Confluent monolayers of sJ-1 NIH 3T3 cell and insert-less vector NIH 3T3 cell transfectants were lysed by scraping in 1 ml of SDS PAGE sample buffer containing 2% (v/v) mercaptoethanol and boiled for 10 min. To equalize for protein load, cells were independently lysed by scraping into 20 mM Tris buffer pH 7.5 containing 1% (v/v) Triton X100, the protein concentration measured using the Coomassie Protein Assay Kit (Pierce), equal protein loads resolved by 6% acrylamide (w/v) SDS PAGE, transferred to Hybond C membranes (Amersham) and blotted with the SP1.D8 monoclonal antibody to the pro-α-1(I) collagen amino-terminal extension peptide (Developmental Studies Hybridoma Bank, University of Iowa). Pro-α-1(I) collagen was visualized using a horseradish peroxidase-conjugated goat antibody against mouse IgG (Bio-Rad) and the ECL detection system (Amersham).

Cell culture dishes were coated with 10 μg/cm^2 of human fibronectin for 2 h, the fibronectin removed and the plates washed three times with sterile PBS. Collagen gels were formed in 6 well plates by mixing type I collagen (Vitrogen 100, Collagen Corporation), 10× DMEM (Gibco/BRL), and sodium bicarbonate (11.8 mg/ml) in a 8:1:1 (v/v) ratio on ice and then quickly dispensed (1.5 ml) into the individual cell culture dishes. The collagen mixture was allowed to gel for 1 h prior to use. Soft agar growth assays were performed as previously described (12).

Serial analysis of gene expression (SAGE). The SAGE method was performed as previously described (13, 14). Briefly, polyA$^+$ RNA derived from insert-less vector control and sJ-1 NIH 3T3 cell transfectant-derived polyA$^+$ RNA were converted to ds-cDNA (cDNA Synthesis System, BRL) using 5′-biotinyl-dT$_{18}$ (Integrated DNA Technologies, Inc.). The cDNA was cleaved with NlaIII, the 3′-biotinylated fragments captured on streptavidin-coated magnetic beads (Dynal), the bound cDNA was divided into two pools, and one of the following linkers containing recognition sites for BsmFI and a NlaIII complementary terminus was ligated to each pool: linker 1, 5′-TTTGGATTTGCTGGTGCAGTACAACTAGGCTTAATAGGGAC-ATG-3′, 5′-TCCCTATTAAGCCTAGTTGTACTGCACCAGCAA-ATCC (amino-C7)-3′ and linker 2, 5′-TTTCTGCTCGAATTC-AAGCTTCTAACGATGTACGGGGACATG-3′, 5′-TCCCCGTACATC-GTTAGAAGCTTGAATTCGAGCAG (amino-C7)-3′. SAGE tags were released with BsmFI, the tag overhangs filled in with T7 polymerase, ligated using T4 DNA ligase (BRL) overnight at 25°C, diluted and amplified by PCR for 28 cycles (primers: 5′-GGATTTGCTGGT-GCAGTACAACT-3′ and 5′-CTGCTCGAATTCAAGCTTCTAAC-3′). The product was fractionated by PAGE, the 104 bp product containing two tags ligated tail to tail (ditag), excised and extracted from the gel, cleaved with NlaIII, and the ditags purified by gel electrophoresis, excised and self-ligated to produce ditag concatamers (13,14). The concatenated products were separated by PAGE, products between 300 bp and 800 bp were excised and cloned into the SphI site of pZero (Invitrogen). Colonies were screened for insert size by PCR with M13 forward and M13 reverse primers. Clones were introduced into 25 μl PCR reactions containing 0.5 μM M13 forward and reverse primers and subjected to thermal cycling (25 cycles) consisting of 20 sec at 95°C, 1 min at 52°C, and 1 min at 72°C. Clones selected on the basis of insert size were subjected to automated fluorescent DNA sequence analysis using rhodamine dideoxynucleotide terminator chemistry according to the instruction of the manufacturer (Applied Biosystems, Inc.).

Sequence files were analyzed by means of the SAGE program group, which identifies the anchoring enzyme site with the proper spacing, extracts the two intervening tags, and records them in a database. The potential identities of the tags were established by their presence in GenBank or DbEST databases (release 109).

Assessment of soluble jagged-1 NIH 3T3 cell transfectant behavior in vivo. The sJ-1 NIH 3T3 cell transfectants were grown to confluence under G418 selection and 24 h prior to injection the medium was changed to DMEM containing 10% (v/v) FBS. The transfectants were washed with PBS, harvested by trypsin digestion and resuspended in sterile/pyrogen-free PBS prior to injection. The cells were >95% viable by trypan blue exclusion and were free of mycoplasma and indigenous murine viruses including mouse hepatitis, adenovirus, pneumonia, cytomegalovirus, and Sendai (Anmed/Biosafe Inc, Rockville, MD). Female athymic nude mice (nu/nu) between 8–12 weeks of age (NCI-FCRDC) received 150 mg/kg of cyclophosphamide in pyrogen-free water by the intraperitoneal route 24 h prior to injection and 200 μl of cell suspension (10^6 cells) was injected intradermally into the right flank. Following euthanasia, tissue growths were exposed by dissecting along the subcutaneous tissue plane and the tissue masses removed, fixed in 10% (v/v) buffered formalin and processed for paraffin sectioning and hematoxylin and eosin stain-

TABLE 1

Most Frequently Observed SAGE Tags

RNA Source	Tags Sequenced	Discrete Tags	mRNA Species
Insert-less Vector	1428	982	197
Soluble Jagged-1	3150	1647	336
Totals	4578	2629	533

Tag	Count	Accession	Description
		Tags Predominant in Soluble Jagged-1 NIH 3T3 Cell Transfectants	
TGGATCAGTC	14	M62952	Mus musculus ribosomal protein L19
TAAAGAGGCC	9	U67770	Mus musculus ribosomal protein S26 (RPS26) mRNA
CCTGATCTTT	8	X06406	Mouse mRNA for translational controlled 40 kDa protein
TGTAACAGGA	8	X04648	Mouse mRNA for IgG1/IgG2b Fc receptor (FcR).
TCTGTGCACC	6	U93864	Mus musculus ribosomal protein S11 mRNA
CCAAATAAAA	6	U13687	Mus musculus DBA/2J lactate dehydrogenase-A
CTAATAAAAG	6	X54691	Mouse COX4 mRNA for cytochrome c oxidase subunit
GCCAAGGGTC	5	L08651	Mus musculus large ribosomal subunit protein mRNA
GTCTGCTGAT	5	X75313	M. musculus (C57BL/6) GB-like mRNA.
AAGGAAGAGA	4	X51438	Mouse mRNA for vimentin.
TGAAATAAAC	4	M33212	Mouse nucleolar protein N038 mRNA
CACCACCACA	4	X05021	Murine mRNA with homology to yeast L29 ribosomal prot.
CCTCAGCCTG	4	X52886	M. musculus mRNA for cathepsin D.
CTCTGACTTA	4	Y16256	Mus musculus mRNA for basigin
GTGGGCGTGT	4	M33330	Mouse insulinoma (rig) mRNA
TCCTTGGGGG	4	U60001	Mus musculus protein kinase C inhibitor (mPKCI) mRNA
		Tags Predominant in Control Insert-less Vector NIH 3T3 Cell Transfectants	
CGCCTGCTAG	3	X58251	Mouse COL1A2 mRNA for pro-alpha-2(I) collagen.
AAAAAAAAAA	2	AF0253	Mus musculus tssk-1 and tssk-2 kinase substrate mRNA
AAGCAGAAGG	2	M16465	Mouse calpactin I light chain (p11) mRNA complete
CAGGACTCCG	2	M26270	Mouse stearoyl-CoA desaturase (SCD2) mRNA
GAAGCAGGAC	2	D00472	Mouse mRNA for cofilin
GGATATGTGG	2	M20157	Mouse Egr-1 mRNA
GTTCTGATTG	2	U88588	Mus musculus cdr2 mRNA

Note. Tags correspond to the 10 base pairs of DNA sequence data immediately following the NlaIII cleavage site. The count refers to the number of instances the tag appears in the SAGE database. Accession numbers are the GenBank designations referring to the mRNA identified in the description column. SAGE was conducted on cDNA derived from NIH3T3 cells that had been stably transfected with the pMexNeo insert-less parent vector or the sJ-1 construct. A total of 4578 Tags were sequenced consisting of 1428 from pMexNeo and 3150 pMexNeo sJ-1-transfected cell derived cDNA. Analysis of the data revealed a total of 2629 discrete tags comprised of 982 separate mRNA species from pMexNeo and 1647 separate mRNA species from the sJ-1-transfected cell-derived cDNA. Linkage to GenBank database version 109 yielded a total of 533 matches with documented mouse mRNA species composed of 197 mRNA species from the pMexNeo-derived tags and 336 mRNA species from the sJ-1-derived tags. A p-value of 0.05 or less was chosen as the cutoff for statistically relevant alterations and only the most predominant tags are shown.

ing. Representative portions of these masses were also embedded in O.C.T. compound (Miles Scientific, Elkhart, IN) and snap frozen in 2-methylbutane (E.M. Science, Gibbstown, NJ) on dry ice. Frozen sections were placed onto glass slides, fixed in chilled acetone and dried. Immunohistochemistry was performed using the ABC system (Vector) and a 1:200 dilution of a rat-derived anti-CD31/PECAM (Pharmingen) and the chick chorioallantoic membrane (CAM) angiogenesis assay was performed as previously described (15, 16).

RESULTS

SAGE analysis of sJ-1 NIH 3T3 cell transfectants. The sJ-1 transfectants were analyzed for J-1 expression by immunoprecipitation of [^{35}S]-cys/met-labeled cells and SDS-PAGE analysis of the myc epitope immunoprecipitants resolved a band of approximately 130 kDa in both cell lysate and conditioned medium which corresponds to the size predicted by the mass of the sJ-1 myc epitope translation product (data not shown). Analysis of differential gene expression by SAGE also revealed that the sJ-1 transfectants were able to differentially express 227 transcripts containing either known or novel sequences. These results have been posted (http://Zappa.mmcri.mmc.org/~varyc/jag) and the most prevalent identifiable tags are listed in Table 1. Among the 163 known transcripts expressed by the sJ-1 NIH 3T3 cell transfectants were cathepsin D (Z53337), vimentin (X51438), and among

the 64 known transcripts with apparent reduced levels of expression was pro-α-2(I) collagen (X58251). Because SAGE analysis can provide insight into the presence of known metabolic or signaling pathways, we noted that the sps1/ste20-related kinase, YSK2 (U49949), enhancer of split-Graucho, ESG (X73360), Mus musculus protein kinase C inhibitor, mPKCI (U6001), type IV collagenase (X83424) and connexin (M63802) were present in the sJ-1 NIH 3T3 cell transfectants whereas the fibroblast growth factor receptor-1 (M33760) and IκB-β (U19799) transcripts were not.

Since pro-α-2 (I) collagen expression appeared to be prominent among the repressed transcripts, we attempted to study the expression of the translation product by insert-less vector and sJ-1 NIH 3T3 transfectants. However, this study was limited to the expression of the pro-α-1(I) collagen translation product since antibodies specific for pro-α-2(I) collagen are not available. As shown in Fig. 1B, it was possible using immunoblot analysis to readily detect the pro-α-1(I) collagen translation product in the insert-less vector NIH 3T3 cell transfectants but we were unable to detect the expression of this protein in two stable clones of the sJ-1 NIH 3T3 cell transfectants.

NIH 3T3 cell sJ-1 transfectants exhibit the formation of a matrix-dependent chord-like phenotype. Since collagen matrixes are known modifiers of cellular phenotype *in vitro* (17), we plated the sJ-1 transfectants on type I collagen. As shown in Fig. 2, the sJ-1 transfectants exhibited a chord-like phenotype with the formation of an interlacing arborizing pattern. This chord-like phenotype was also observed when the sJ-1 transfectants were plated on plastic at low cell seed density (Fig. 2) in which groups of cells organize into chord-like arrays one to two cells in width. While these structures progress through the arboring phase, the monolayer assumes a normal NIH 3T3 cell phenotype as the population density nears confluence. In contrast, sJ-1 transfectants did not exhibit a chord-like phenotype on either fibrin, fibronectin, or vitronectin-coated surfaces (data not shown). Likewise, neither wild-type NIH 3T3 cells (Fig. 2) nor insert-less vector NIH 3T3 cells transfectants exhibit this chord-like phenotype either on plastic or on a type-I collagen matrix.

NIH 3T3 cell sJ-1 transfectants modify angiogenesis in vivo. A comparative assessment of the proliferative potential of the sJ-1 transfectants with insert-less vector transfectants revealed that the population doubling time was not altered when cells were subconfluent and this was consistent with the absence of a transformed *in vitro* phenotype including the failure of the sJ-1 transfectants to grow in soft agar (data not shown). However, the sJ-1 transfectants were not sensitive to contact inhibition of growth. As shown in Fig. 1B, the

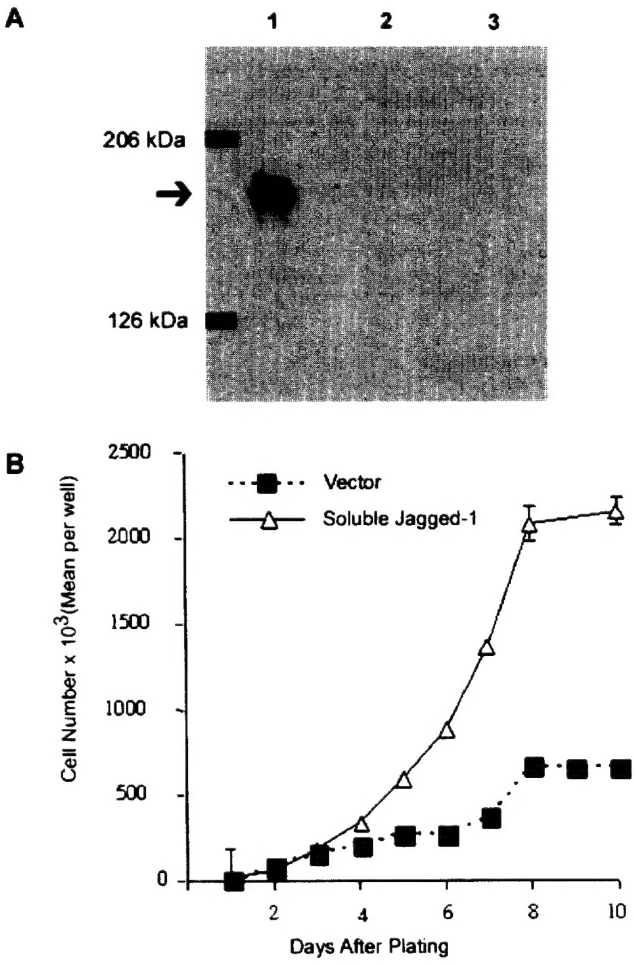

FIG. 1. (A) Immunoblot analysis murine pro-α-1(I) collagen expression in insert-less vector and sJ-1 NIH 3T3 cell transfectants. Cell lysates were prepared from pMEXneo insert-less control NIH 3T3 cell transfectants (lane 1) and sJ-1 NIH 3T3 transfectant clones 38-1 and 38-4 (lanes 2 and 3) and immunoblot analysis of pro-α-1(I) collagen performed as described in Materials and Methods. (B) Growth kinetics of sJ-1 and insert-less vector NIH 3T3 cell transfectants. Cells were plated at a cell seed density of 1×10^4 cells/cm^2 and counted daily in quadruplicate. Both insert-less vector and sJ-1 populations reach confluence at approximately four days after plating. Data are reported as the mean ± standard error of the mean.

sJ-1 NIH 3T3 cell transfectants exhibited the ability to grow to significantly higher cell densities that the insert-less vector NIH 3T3 cell transfectants. Because of this difference, we assessed their potential to form tumors in athymic nude mice and observed that the sJ-1 transfectants were able to form tissue masses (Fig. 3A) but only after an extended latency period of approximately 8 weeks. Full necropsy of these animals did not reveal any evidence of local or distant metastases and gross dissection of these tissue masses revealed prominent angiogenesis characterized by 1–2 large feeder vessels (Fig. 3A). While histologic examination further revealed large numbers of capillaries on

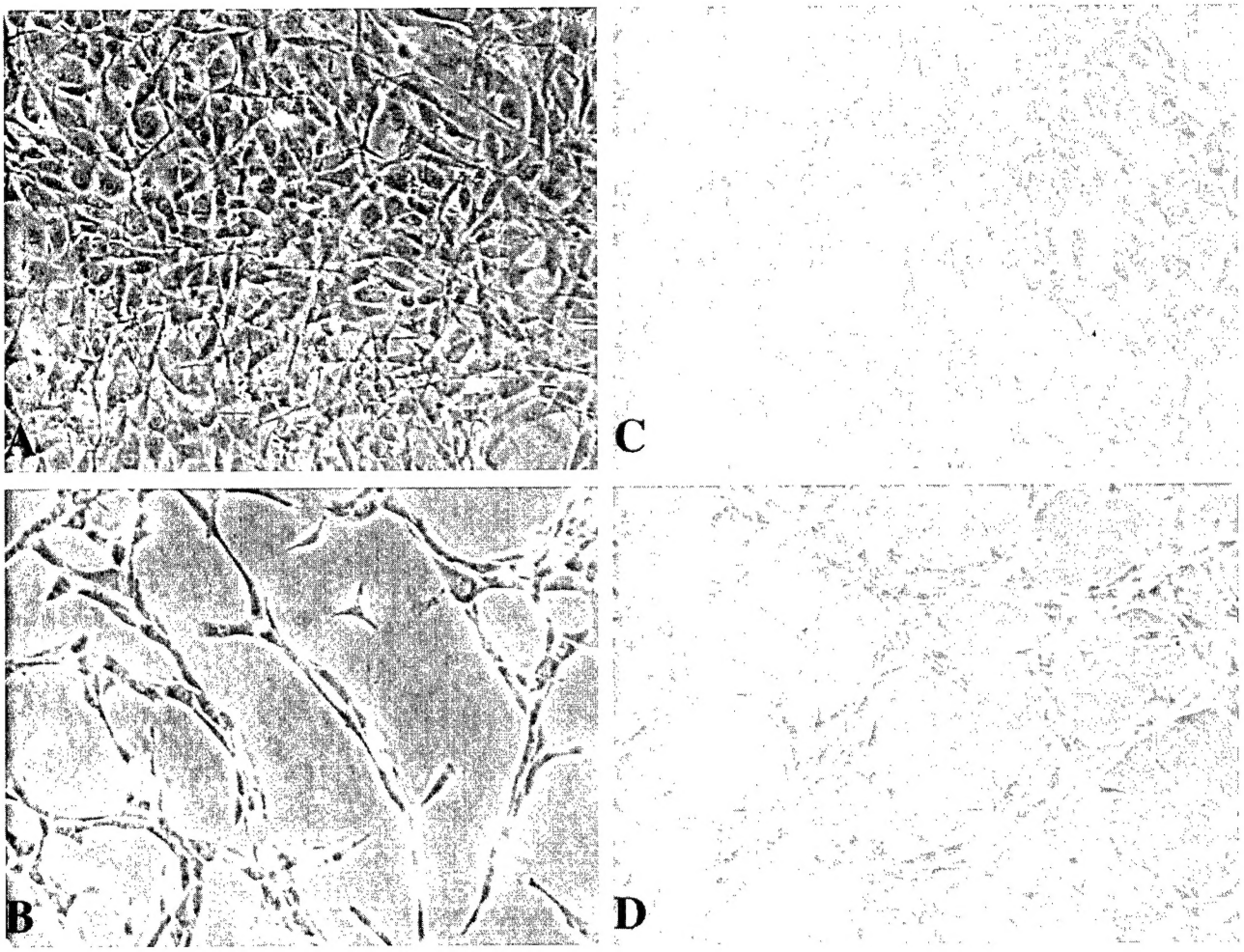

FIG. 2. Formation of chords by sJ-1 transfected cells. Insert-less vector control NIH 3T3 cell transfectants (A, C) and sJ-1 NIH 3T3 cell transfectants (B, D) were plated at 2×10^4 cells per cm^2 on either cell culture plastic (A, B) or on type I collagen (C, D). Two days after plating, the sJ-1 NIH 3T3 cell transfectants formed multicellular chords on plastic (B) and collagen (D). Phase contrast ×100.

the surface that penetrated into the body of the tissue (Fig. 3B), immunohistochemical analysis of the endothelial cell-specific marker, CD31 (PECAM), revealed not only the presence of microvessels but also a plethora of CD31-positive cells organized as a collection of either noncontiguous single cells or sharply angulated short linear arrays (Figs. 3C and 3D). Interestingly, unlike the well-formed intratissue mass microvessels (Fig. 3B), very few of these groups of CD31-positive cells contained blood, nor were they associated with intratissue mass blood spaces (Figs. 3C and 3D). Primary *in vitro* cell isolates of the sJ-1 NIH 3T3 transfectants obtained from these tissue masses by G418 selection also demonstrated their ability to form chord-like structures and re-implantation into nude mice demonstrated their ability to develop angiogenic tissue masses with a similar latency period (data not shown). In addition, the sJ-1 NIH 3T3 transfectants yielded a prominent angiogenic response in the conventional chorioallantoic membrane assay (data not shown).

DISCUSSION

Although we identified the human J-1 transcript as a gene modified during the early stage of *in vitro* angiogenesis (19), the data reported here suggest a role for the soluble form of J-1 as a modifier of chord formation. Interestingly, SAGE analysis suggests alterations in gene expression that may be relevant to the function of J-1 during cell differentiation *in vitro*. In addition to the repression of the type I collagen gene expression, the steady state levels of the transcripts for FGFR-1 and IκB are also reduced. Since (i) J-1 appears to be involved in the regulation of cell differentiation (6), (ii) FGFR-1 signaling is antagonized by effectors which promote differentiation such as γ-interferon, PMA, IL-1, and TNF (20,21), and (iii) many of these modifiers of differentiation are involved in NFκβ-mediated signaling (22), it is possible that J-1-mediated Notch signaling may be involved in regulating these events. This suggestion is consistent with the up-regulation of en-

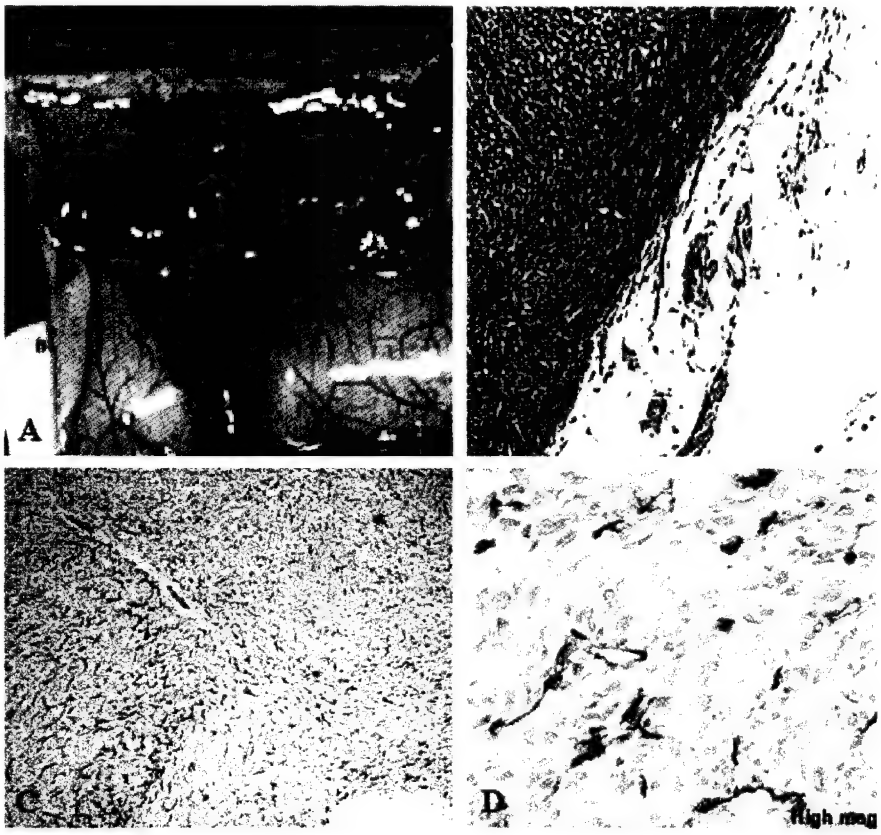

FIG. 3. sJ-1 tissue mass formation in nude mice. (A) Deep dermal view of a sJ-1 NIH 3T3 cell tissue mass 10 weeks after intradermal injection into the flank of a nude mouse. Note the prominent angiogenesis and the arborizing microvessels over the deep surface. (B) Hematoxylin and eosin stain of a paraffin section of the same tumor showing the prominent surface blood filled capillaries, penetrating vessels, and intra-tumor blood islands; magnification is 100×. (C) Low magnification view (100×) of a frozen section of the same tumor showing immunohistochemical localization of CD31 (PECAM). Two cross sections of a microvessel are evident along with a high density of CD31 positivity which, upon higher (500×) magnification (D) is comprised of groups of single cells or angulated collection of CD31-positive cells.

hancer of split-Groucho, a known component of Notch signaling (23). Likewise, connexin, which plays an important role in the formation of tight cell to cell contacts (24), may be modified during J-1-dependent chord development. It is also interesting that, like connexin, the increase in the expression of the type IV collagenase transcript may be relevant to the differentiation process since it is well established that proteolytic modification of collagen matrixes is a component of the migratory phenotype (25) during the process of chord development. In addition, these alterations in gene expression mediated by J-1 may also be involved in directing the formation of a chord-like phenotype during the organization component of the non-terminal endothelial cell differentiation pathway (26). While transmission electron microscopic analysis of the chord-like structures revealed prominent interdigitations between cells with close membrane apposition (data not shown), a distinct lumen with interdigitation of the plasma membrane was not readily observed despite their resemblance to the tubular phenotype observed with *in vitro* populations of endothelial cells (27). We suggest that absence of a readily visible lumen in the chords formed by the sJ-1 NIH 3T3 cell transfectants may be either a consequence of another gene product, the absence of appropriate rheologic conditions or the absence of another genomic requisite not present in the NIH 3T3 cell. We further suggest that it is likely that another gene product may be responsible for lumen formation since the majority of the CD31-positive chord-like structures established in the sJ-1 tissue masses *in vivo,* also do not exhibit evidence of blood flow.

These data are consistent with the recent genetic observation that the J-1 null mouse exhibits normal vasculogenesis but an abnormal and early lethal embryonic angiogenic phenotype including defects in the remodeling of the yolk sac and embryonic vasculature (26). Indeed, the vascular pathology apparent in the J-1 null mouse (26) may be related to their inability to modulate the chord development component of the endothelial cell differentiation pathway (27). Likewise, the observation that mutations in the human Notch-4 gene are responsible for the formation of CADASIL, a

systemic vascular disease (28), is also consistent with the concept that Notch signaling is an important component of vascular physiology in man. It is also noteworthy that the observation relating the repression of J-1 function in human endothelial cells to an exaggeration of the ability of FGF but not VEGF to induce sprout formation (19) also correlates well with the role of VEGF but not FGF as a mediator of vasculogenesis since the J-1 null mice exhibit hemorrhage as a result of the failure to form the large vitelline blood vessels, a process mediated by angiogenesis (26). We suggest that this defect may ultimately involve enhanced endothelial cell sprout formation and a failure of the mutant vasculature to form chords.

The function of the ectodomain of J-1 as a biological response modifier is also consistent with the recent observation (29) that the enzymatic function of kuzbanian, an ADAM metalloprotease gene family member (30), is required for the activity of the *Drosophila* Notch ligand, Delta. Although it is not known whether a proteolytic modification of the *Drosophila* J-1 homolog, Serrate (31), requires a similar proteolytic modification, our data do suggest that the ectodomain of J-1 may function in the absence of its transmembrane domain as an extracellular protein. Although we attempted to utilize medium conditioned by the sJ-1 NIH 3T3 cell transfectants as a resource for the transfer of this protein to other cell culture systems, we were unable to do so. While we do not know the reason for this inefficiency, it may be due to the lability of the sJ-1 translation product in the conditioned medium.

ACKNOWLEDGMENTS

The authors thank A. Blier for expert administrative assistance and Dr. Heinz Furthmayr, Stanford University for the antibody against procollagen type I. This research effort was supported by Grants HL32348 and AG07450 from the NIH and the U.S. Army Breast Cancer Research Program to T.M. This manuscript is dedicated to the memories of Werner Risau and Russell Ross, two friends and colleagues.

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To: J. Biol. Chem., Rapid Communication, 2000

Src-Dependent Chord Formation Mediated by Jagged 1 Is Independent of Cell Migration

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Acknowledgements. The authors thank Robert Friesel and Doug Spicer (Maine Medical Center) for providing the dnSrc, MyoD and MCK luciferase constructs, the laboratory of Thomas Kadesch (University of Pennsylvania) for confirmation of the HES1 reporter gene data, the officers of Takeda Pharmaceuticals, Ltd. for amlexanox, and Alan Kraker (Parke Davis) for supplying the Src-specific inhibitor, PD0180970. The authors also thank Julia Frothingham and Norma Albrecht for expert administrative assistance. This effort was supported in part by NIH Grants HL35627, RR15555 and AG07450 to TM.

Abbreviations: BCS, Bovine Calf Serum; DMEM, Dulbecco's Modified Eagle's Medium; DN, Dominant Negative; FGF, Fibroblast Growth Factor; FGFR, FGF Receptor; HES, Hairy Enhancer of Split; MCK, Muscle Creatine Kinase; N1IC, Constitutively Active Notch 1; PBS, Phosphate-Buffered Saline; PK, Protein Kinase; SDS-PAGE, Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis; sJ1, Soluble Jagged 1

Key Words: Jagged, Notch, Src, Collagen Type I, Cortactin and Amlexanox

Running Title : Jagged 1-Mediated Chord Formation

ABSTRACT

The expression of the extracellular domain of the transmembrane ligand, Jagged 1 (sJ1), in NIH 3T3 cells results in the formation of a Collagen Type I-dependent chord-like phenotype reminiscent of the phenotype exhibited by endothelial cells during the mid-phase of their differentiation pathway. In an effort to determine the mechanism by which sJ1 induces this phenotype, we observed that sJ1 transfectants display an increase in the activity of Src. Cotransfection of sJ1 transfectants with a dominant negative mutant of Src resulted in the loss of matrix-dependent chord formation and correlated with the restoration of Collagen Type I expression and contact inhibition of growth; events which are absent in the sJ1 transfectants. Interestingly, amlexanox, an agent which inhibits cell migration and decreases the abundance of actin stress fibers, was unable to prevent Collagen Type I-dependent chord formation. These data suggest that the ability of sJ1 to mediate chord formation is dependent upon Src activity and is tolerant to the destabilization of the actin cytoskeleton, a mediator of cell migration.

INTRODUCTION

Notch receptors are transmembrane polypeptides that regulate cell fate determination and whose activities are critical for a wide variety of developmental and physiological processes (1,2). Notch genes have been found in all invertebrate and vertebrate species examined to date with four Notch genes (Notch1-4) expressed in mice, rats and humans (3-6). Notch ligands are also evolutionarily conserved transmembrane polypeptides that are divided into Delta-like or Serrate-like subclasses based on the absence or presence, respectively, of an extracellular cysteine-rich region located NH_2-

terminal to the transmembrane domain (7). Four Delta-like (Delta 1-4) and two Serrate-like (Jagged 1 and 2) genes have thus far been identified in vertebrates (8-10).

We have previously reported that the Notch ligand, Jagged 1 was upregulated in human endothelial cells during the fibrin-mediated pathway of *in vitro* angiogenesis (11,12). Indeed, Jagged 1 expression is mandatory for normal blood vessel formation since embryonic mice are not viable beyond stage E10.5 in a Jagged 1 null situation, presumably as a result of massive hemorrhage associated with malformation of the vasculature (13). During studies to define role of Jagged 1 signaling in angiogenesis, we noted prominent phenotypic alterations in cells stably transfected with a naturally occurring and secreted form of Jagged 1 lacking the transmembrane and intracellular domains (14). Indeed, stable soluble Jagged 1 (sJ1) NIH 3T3 cell transfectants exhibited a wide range of biochemical, morphological and behavioral changes that include loss of Collagen Type I expression, decreased sensitivity to contact inhibition, and the matrix-dependent formation of chord-like structures (14), a morphologic characteristic reminiscent of the early-to-mid stage phenotype exhibited during the pathway of human endothelial cell differentiation (15). The role of Jagged 1 as an angiogenic factor was further substantiated by the observation that the implantation of lethally irradiated sJ1 NIH 3T3 cell transfectants in the conventional chorioallantoic membrane assay induced the growth of neovessels (14).

While the signaling pathways initiated by sJ1 that lead to these phenotypic changes are unknown, it is likely that they involve the activation of Notch receptors. Extensive genetic and biochemical studies in both invertebrates and vertebrates have demonstrated that activated Notch mediates many of its effects by upregulating the

expression of the hairy enhancer of split (HES) family of transcription factors after forming a complex with CSL (CBF1/RBP-Jk/KBF2 in mammals; Su(H) in *Drosophila* and *X. laevis* and Lag2 in *C. elegans*) proteins(16,17). In contrast, there is increasing evidence which suggests that Notch may induce the activation of signaling pathways that are independent of CSL/HES1 interactions (18,19).

In an effort to elucidate the signaling mechanisms responsible for the sJ1-inducible chord-like phenotype, we examined the role of protein tyrosine phosphorylation in this system. We report that sJ1 NIH 3T3 cell transfectants demonstrate a pattern of protein tyrosine phosphorylation indicative of an increase in the activity of Src and the expression of a dominant negative (dn) form of Src in the sJ1 NIH 3T3 cell transfectants was able to revert matrix-dependent chord formation including a down-regulation of F-actin stress fibers, an observation consistent with the activation of Src (20). In addition, treatment with amlexanox, an agent known to repress cell migration and attenuate the F-actin cytoskeleton (21), was unable to repress chord-formation suggesting that cell migration is not requisite for chord formation *in vitro*.

EXPERIMENTAL PROCEDURES

Cell Culture, Transfection and Immunoblot Analysis: Clone 38-1 representing NIH 3T3 cells transfected with the soluble form of Jagged 1 (sJ1) and control NIH3T3 cells transfected with the insert-less vector pMEXneo (14) were used in the present study. Cells were grown in DMEM (GibcoBRL) supplemented with 10% (v/v) bovine calf serum (BCS; HyClone), 100 U/ml penicillin G, 100 µg/ml streptomycin, 0.25 µg/ml fungizone (GibcoBRL) and 400 µg/ml geneticin (GibcoBRL). The sJ1 NIH

3T3 cell transfectants were cotransfected with a dominant negative (dn) mutant of *X. laevis* Src in which the lysine 294 was replaced by alanine and the tyrosine 526 by phenylalanine. The dnSrc mutant was cloned into pcDNA 3.1/Hygro vector (Invitrogen) using Xho I and Xba I restriction sites and the selection of stable transfectants was performed as previously described (22). Control transfection was performed using the insert-less pcDNA 3.1/Hygro vector. The expression of the dnSrc construct was confirmed by RT-PCR analysis using two Src primers one of which was specific for specific for *X. laevis* Src and by immunoblot analysis of cell lysates with Src2 antibodies (Santa Cruz). Clone dn3 representing the dnSrc:sJ1 NIH 3T3 cell cotransfectants was used for these studies. Cells were made quiescent by incubating the confluent monolayer in serum-free DMEM containing 10μg/ml insulin (DMI) as described (22) for 48 h and further stimulated by addition of 10 mg/ml heparin (Upjohn) and 10 ng/ml recombinant human FGF1, prepared as previously described (23). Immunoprecipitation of cortactin was performed as described (24) while immunoblot analysis of total phosphotyrosine and Src utilized antibodies from Upstate Biotech and Santa Cruz, respectively, using methods previously described (24). Collagen Type I (SP1.D8 monoclonal antibody from the Developmental Studies Hybridoma Bank, University of Iowa) and cortactin (25) immunoblot analyses utilized similar methods . Amlexanox (AA673; Takeda Chemical Industries, Osaka, Japan) was dissolved in equimolar NaOH as described (26) and the Src inhibitors PP2 (Sigma) and PD0180970 (Parke-Davis) were dissolved in dimethylsulfoxide.

Fluorescence Microscopy, Chord Formation, Cell Migration and Growth: Immunofluorescence staining was performed as previously described (24). Briefly, cells

were plated on glass coverslips. After 24 h, the cells were fixed with 4% (v/v) paraformaldehyde in PBS for 5 min at 25°C, the monolayers washed with PBS and blocked with PBS containing 5% (w/v) BSA, 0.1% (v/v) Triton X-100, 0.1% (v/v) Tween 20 and 0.1% (w/v) NaN_3 (blocking buffer) for 1 hour. For F-actin staining, the cell monolayer was washed with PBS three times and incubated for 20 min with 0.1 μg/ml CY3-conjugated phalloidin (Sigma), washed three times in PBS and embedded in 50% (v/v) glycerol containing 0.1% (w/v) phenylenediamine. Cells were studied using the fluorescent confocal microscope, Leica TCS-SP, and pictures of optical sections most abundant in fluorescent F-actin were recorded.

To study substrate-dependent chord formation, cells were plated on either Collagen Type I or on fibronectin. Cell culture dishes were coated with 10 $\mu g/cm^2$ of human fibronectin prepared as previously described (27). Collagen gels were formed in six well cell culture dishes by mixing Type I collagen (Becton Dickinson), 10X PBS, 1N NaOH and sterile distilled H_2O in a 2.5:1:0.06:6.5 (v/v) ratio on ice and then quickly dispensed (1.5 ml) into the individual wells. The collagen mixture was allowed to gel for 1h prior to use. For studies involving cell migration, confluent monolayers were scraped and the response to injury recorded by phase contrast microscopy as previously described (24). Cell growth was examined by seeding cells in complete culture medium on fibronectin-coated dishes ($10mg/cm^2$) at an initial density of 10^4 cells per well. Cell numbers were monitored every 2 days, using trypsin/EDTA cell detachment and quantitation using a hematocytometer as described (14).

Transient Transfection Assay of MyoD -Mediated Transcription of a Luciferase Reporter Gene: Insert-less, pMEXneo and sJ1 NIH 3T3 cell transfectants

were plated into twelve well cell culture dishes containing DMEM and 10% (v/v) BCS and transiently transfected with 300 ng CMV-MyoD and 500 ng luciferase reporter construct driven by the muscle creatine kinase (MCK) promoter (28). Some samples were also transfected with either 300 ng of a constitutively active Notch 1 construct (N1IC) prepared as described (29), 500 ng of sJ1 (14) or a combination of both N1IC and sJ1. Transfection was performed using Fugene 6 (Boehringer Mannheim) and 24 h after transfection, the growth media was changed to DMEM containing 0.5% (v/v) BCS, the cells incubated for an additional 48 h and the cells harvested and measured for luciferase (Luc) activity using Promega's Dual-Luciferase Reporter assay system per manufacturer's instructions. The efficiency of transcription was measured in relationship to the activity of pRL-SV40 *Renilla* (Promega) and normalized to protein concentration as described (28). Activity was reported as fold-induction over background as determined by the activity of cells transfected with MCK-Luc reporter, only.

RESULTS AND DISCUSSION

The Expression of Soluble Jagged 1 Mediates the Tyrosine Phosphorylation of Cortactin and an Attenuation of F-Actin Stress Fibers *In Vitro*. In an attempt to elucidate the potential role of tyrosine kinases as possible mediators of sJ1-dependent function, the protein tyrosine phosphorylation profiles of whole cell lysates obtained from insert-less pMEXneo and sJ1 NIH 3T3 cell transfectants were examined by anti-phosphotyrosine immunoblot analysis. As shown in Figure 1A, the steady-state levels of phosphotyrosine-containing proteins were significantly different between the insert-less vector and sJ1 NIH 3T3 cell transfectants. Interestingly, we observed the presence of two

prominent phosphotyrosine-containing polypeptides with apparent molecular masses of approximately 60 kDa and 80 kDa in the sJ1 NIH 3T3 cell transfectants (Figure 1A). Since these bands could possibly represent the phosphotyrosine signature of Src and its substrate, the F-actin-binding protein, cortactin (22,25), we examined this possibility using cortactin immunoprecipitation followed by phosphotyrosine and cortactin immunoblot analysis in sJ1 NIH 3T3 cell transfectants in response to FGF1, a known inducer of Src-dependent cortactin tyrosine phosphorylation (22,25) in the presence and absence of PP2, a Src-specific tyrosine kinase inhibitor (30). As shown in Figure 1B, the intracellular levels of cortactin did not vary in response to either FGF1 or PP2. However, cortactin displayed an increase in tyrosine phosphorylation in quiescent sJ1 NIH 3T3 cell transfectants which was further exaggerated by FGF1 stimulation and repressed by the addition of PP2 (Figure 1B). Further, immunoprecipitation of the cell lysates with an anti-cortactin antibody followed by anti-phosphotyrosine immunoblot analysis confirmed the initial observation that sJ1 NIH 3T3 cell transfectants exhibit an increase in the phosphorylation of cortactin (Figure 1B) and these results suggested an increase in Src signaling activity in the sJ1 NIH 3T3 cell transfectants.

Another indication of the enhanced activity of Src was the attenuation of F-actin stress fibers in the sJ1 NIH 3T3 cell transfectants. As shown in Figure 1C, confocal fluorescent microscopy of the cells stained with a fluorescent (CY3) conjugate of phalloidin exhibited a prominent reduction in F-actin stress fibers. Indeed, the down-regulation of actin stress fibers may be explained by the Src-induced tyrosine phosphorylation of cortactin (25) leading to detachment of cortactin from actin microfilaments (31) and thus resulting in dissociation of stress fibers to individual

filaments. It is well documented that the expression of the constitutively active v-Src results in a dramatic down-regulation of actin stress fibers (32).

Cotransfection of a Dominant Negative Src Construct Represses the Formation of the Soluble Jagged 1-Mediated Chord-Like Phenotype. In an attempt to determine whether phenotypic characteristics of the sJ1 NIH 3T3 cell transfectants cells are associated with an increase in Src activity, we cotransfected the sJ1 NIH 3T3 cell transfectants with a dn mutant of *X. laevis* Src containing two point mutations, one which inactivates the catalytic domain, and the other which maintains Src in an activated conformation. Interestingly, the dnSrc:sJ1 NIH 3T3 cell cotransfectants displayed a dramatic decrease in the phosphorylation of cortactin (data not shown), enhanced cell spreading on plastic (Figure 2A), an increase in the level of F-actin stress fibers (Figure 1C) and a repression of the chord-like phenotype in response to Collagen Type I (Figure 2A). Likewise, the addition of, PD0180970, a novel and highly specific inhibitor of Src, was also able to repress chord formation in the sJ1 NIH 3T3 cell transfectants (data not shown).

The expression of dnSrc in the sJ1 NIH 3T3 cell transfectants also reverted the expression of pro-α-1 Type I collagen (Figure 2B) and restored contact inhibition of cell growth (Figure 2C). The restoration of pro-α-1 Collagen Type I expression in dnSrc:sJ1 NIH 3T3 cell cotransfectants agrees with the results of Frankfort and Gelman who have reported a ten-fold decrease of Collagen Type I mRNA after transfection of NIH 3T3 cells with v-Src (33) and also with the correlation between Src signaling and the transcriptional activation of collagenase 1 expression (34). Also, the loss of contact inhibition is a well documented consequence of the expression of v-Src in a variety of

cell types (35). In contrast, cotransfection of the sJ1 NIH 3T3 cell transfectants with an insert-less vector did not alter any of the phenotypic characteristics displayed by sJ1 NIH 3T3 cell transfectants (data not shown). These data suggest that several sJ1-mediated characteristics including chord formation may be attributed to the function of Src and implicates a role for Src in the regulation of Notch-dependent signaling events.

The Chord-Like Phenotype Exhibited by Soluble Jagged 1 NIH 3T3 Cell Transfectants Is Resistant to Amlexanox and an Attenuation of Cell Migration. Because sJ1 expression attenuates the expression of F-actin stress fibers in NIH 3T3 cells, we questioned whether chord formation could be stimulated by the down-regulation of the F-actin cytoskeleton. To examine this premise, we treated insert-less pMEXneo and sJ1 NIH 3T3 cell transfectants with amlexanox, a drug that down-regulates the abundance of F-actin stress fibers without destroying F-actin filaments (21). While amlexanox was able to abolish the presence of the majority of actin stress fibers in NIH 3T3 cells (21), it failed to induce chord formation on Collagen Type I by insert-less pMEXneo NIH 3T3 cell transfectants (Figure 3A). Interestingly, however, amlexanox was not able to repress the formation of the Collagen Type I-dependent chord-like phenotype exhibited by the sJ1 NIH 3T3 cell transfectants. Thus, it appears that the attenuation of actin stress fibers cannot induce substrate-dependent chord formation. In addition, while the dnSrc:sJ1 NIH 3T3 cells cotransfectants displayed an increased resistance to amlexanox-induced destabilization of the F-actin cytoskeleton, these cells did not exhibit a Collagen Type I-dependent chord-like phenotype in the presence of amlexanox (data not shown).

Because the disappearance of actin stress fibers can impair the migratory activity of the cells (36), and amlexanox is a potent inhibitor of cell migration (21), we examined the migratory phenotype of the sJ1 NIH 3T3 cell transfectants. As shown in Figure 3B, the sJ1 NIH 3T3 cell transfectants exhibited a decrease in migration when compared with insert-less pMEXneo NIH 3T3 cell transfectants but the expression of dnSrc did not restore the migratory phenotype. Since the attenuation of stress fibers results in the inhibition of chaotic lateral cell migration (21), it could promote the stabilization of the chord-like phenotype exhibited by the sJ1 NIH 3T3 cell transfectants. Thus, while the activation of Src in the sJ1 NIH 3T3 cell transfectants may provide the necessary changes in the actin cytoskeleton that are essential for the generation of the chord-like phenotype, this signaling pathway may be independent of the pathway utilized by Src to modify cell migration and may require the function of other cellular processes, including the enhancement of specific cell-cell contacts and cell polarization.

CSL/HES-Independent Notch Signaling Pathways Are Active in the Soluble Jagged 1 NIH 3T3 Cell Transfectants. The signaling pathways initiated by sJ1 that lead to phenotypic changes in the NIH 3T3 cell are unknown, but likely involve interactions of the soluble ligand with Notch 1 and/or Notch 2 receptors as transcripts for these, but not Notch 3 and Notch 4 are expressed in NIH 3T3 cells (data not shown). In an attempt to determine if the sJ1 phenotype, including chord formation, results from an increase in Notch activity, we assayed the stable sJ1 NIH 3T3 cell transfectants and NIH 3T3 cells transiently tranfected with sJ1 using two different Notch reporter assays (16,28). As shown in Figure 4, we observed that like the constitutively active mutants of Notch 1 and Notch 2, sJ1 is also capable of quenching MyoD-dependent transcription in

a transient transfection assay that measures MyoD-induced transcription of a luciferase reporter gene linked to the muscle creatine kinase promoter (MCK-Luc). However, it is unlikely that the sJ1-dependent phenotype results from activation of the traditional CSL/HES-dependent Notch signaling pathway as neither NIH 3T3 cells transiently transfected with the sJ1 construct nor stable sJ1 transfectants displayed an increase in CSL-mediated transcription as measured by the activity of a CAT reporter gene linked to the HES1 promoter (data not shown and T. Kadesch, personnal communication). Because several groups have reported that Notch is capable of inducing signaling cascades that inhibit the activity of MyoD independently of CSL/HES1 transcriptional activity (18,19), it is possible that sJ1 activates some, but not all of the Notch-mediated signaling events. Alternatively, the inhibition of MyoD by sJ1 may not be the direct result of Notch activation; rather an indirect consequence of the increased activity of Src. In addition, while the overexpression of v-Src has been reported to interfere with the expression of MyoD and its related family members in cells derived from myogenic lineages (37), it is not known whether Src is able to prevent the ability of the myogenic factors to function in NIH 3T3 cells.

Although the mechanism utilized by sJ1 to increase Src activity is not known, it is possible that sJ1 may enable the Notch receptor to interact via its ankyrin repeats with Deltex, a cytosolic protein which is genetically linked to the Notch null phenotype (38,39). Deltex may, in turn be able to interact with Src through a SH3 domain located within its structure (39) and, interestingly, transfection of cells with Deltex was able to suppress the expression of an E47-responsive CAT reporter known to associate with MyoD (39). Another molecule that may link sJ1-mediated Notch signaling with Src is

Supt6H (EMB-5 in *C. elegans*). EMB-5, a protein implicated in regulating chromatin structure, possibly through mediation of phosphoprotein interactions, interacts with the *C. elegans* Notch receptors Lin-12 and Glp-1 in a yeast two-hybrid system and has been genetically linked to some but not all of cell differentiation events mediated by Notch (40). In contrast to Deltex, this polypeptide contains a SH2 domain that may facilitate interactions between tyrosine kinase and Notch signaling cascades (40). In addition, the carboxy-terminus of the full-length Jagged 1 polypeptide contains a PDZ domain (41) and this may also enable Jagged 1 to be interactive with receptor and non-receptor tyrosine kinase signaling pathways by contact with Notch-positive cells(41).

The observation that amlexanox does not repress the ability of the sJ1 NIH 3T3 cell transfectants to exhibit the formation of a chord-like phenotype in response to Collagen Type I is particularly noteworthy since amlexanox is able to reversibly impair the Src-regulated assembly of F-actin stress fibers and attenuate cell migration *in vitro* (21). Since cell signaling mediated by Src is involved in the regulation of cell migration *in vitro* (42), our data suggest that the role of Src in the formation of chord-like structures may not only be independent of its traditional role as a non-receptor tyrosine kinase modifier of F-actin stress fiber assembly and cell migration but may also be dependent upon a component of the Notch receptor signaling pathway. Because Notch receptor signaling is involved in the determination of cell fate and the ability to form chords is fundamental to the generation of the lumen-forming phenotype (43), out data also imply an unanticipated and divergent role for either Src or members of the Src gene family in these processes and suggest that in a cell density-dependent manner, the migratory phenotype precedes the chord phenotype during the cellular differentiation pathway for

lumen-containing structures. Interestingly, Elicieri, et al. (44) have recently shown that the attenuation of Src activity significantly repressed angiogenesis *in vivo*.

These data may also be relevant to the tubulogenic mechanism of angiogenesis. Indeed, human Jagged 1 was originally isolated as an angiogenic factor from human endothelial cells and endothelial cells treated with an antisense Jagged 1 oligonucleotide exhibit an enhanced migratory sprout-like phenotype in response to FGF but not vascular endothelial growth factor (12). Because Src activity is known to be regulated by FGF in human endothelial cells (45), we anticipate that the signaling pathway regulated by sJ1 may be interactive with the FGFR signaling pathway. Indeed, studies in *Drosophila* with Branchless, the FGF homolog, and Breathless, the FGFR homolog, during tubulogenesis of the trachea have suggested their interaction with Notch-signaling cells (46). In addition, analysis of the murine Jagged 1 and Notch 1 null phenotypes both exhibit early embryonic lethality with profound abnormalities of the vasculature and while the Jagged-1 null mouse exhibits normal vasculogenesis, these mice are deficient in angiogenesis (13). Interestingly, the vascular abnormalities present in the Jagged 1 null mouse are phenotypically similar to those observed in endothelial cells treated with antisense Jagged 1 oligonucleotides (12). Likewise, expression of Jagged 1 and/or a constitutively active form of the Notch 4 receptor, the int3 oncogene (6), has been shown to regulate chord formation in both endothelial (47) and epithelial cells (48) *in vitro* and Notch 4 (6) and Deltex expression (40) have been reported to be exaggerated in vascular tissues. However, because the expression of the constitutive active form of the Notch 4 receptor, int3, in NIH 3T3 cells failed to exhibit any of the phenotypic or biochemical changes mediated by sJ1 (data not shown), it is likely that Notch 4 signaling, by itself, is not

capable of mediating chord formation, at least not in the NIH 3T3 cell. Further, the recent observation that int3 requires the function of Ras-mediated PI-3 kinase but not the function of the protein kinase (PK)- A, PK-C or Src pathways for the initiation of tumor growth (49) also suggests divergent roles for individual Notch receptors that may be coupled to receptor and/or non-receptor tyrosine kinase signaling pathways. Since sJ1 expression results in the formation of a matrix-dependent chord-like phenotype and the regulation of the chord formation is a critical component of lumen formation mediated by endothelial and epithelial cells, it is likely that Notch signaling through a Src-dependent pathway may be involved in the regulation of cellular differentiation in a variety of diverse cell types in which chord formation is a component of their differentiation pathway.

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FIGURE LEGENDS

Figure 1. A. Protein Tyrosine Phosphorylation Patterns in sJ1 and Insert-Less pMexneo NIH 3T3 Cell Transfectants: Total lysates of insert-less pMEXneo and sJ1 NIH 3T3 cell transfectants were prepared and immunoblotted for phosphotyrosine as described in the Experimental Procedures. The protein bands exhibiting differential tyrosine phosphorylation are marked with arrows. **B. Tyrosine Phosphorylation of Cortactin Is Enhanced in sJ1 NIH 3T3 Cell Transfectants:** Cortactin immunoprecipitation and phosphotyrosine (top) and cortactin (bottom) immunoblot analysis from quiescent and FGF 1-stimulated (10ng/ml) insert-less pMEXneo and sJ1 NIH 3T3 cell transfectants was performed in the presence and absence of 10μM PP2, an inhibitor of Src, as described in the Experimental Procedures. **C. Expression of F-Actin Stress Fibers is Attenuated in sJ1 NIH 3T3 Cell Transfectants:** Insert-less pMEXneo (A) and sJ1 NIH 3T3 cell transfectants (B) and dnSrc:sJ1 NIH 3T3 cell cotransfectants

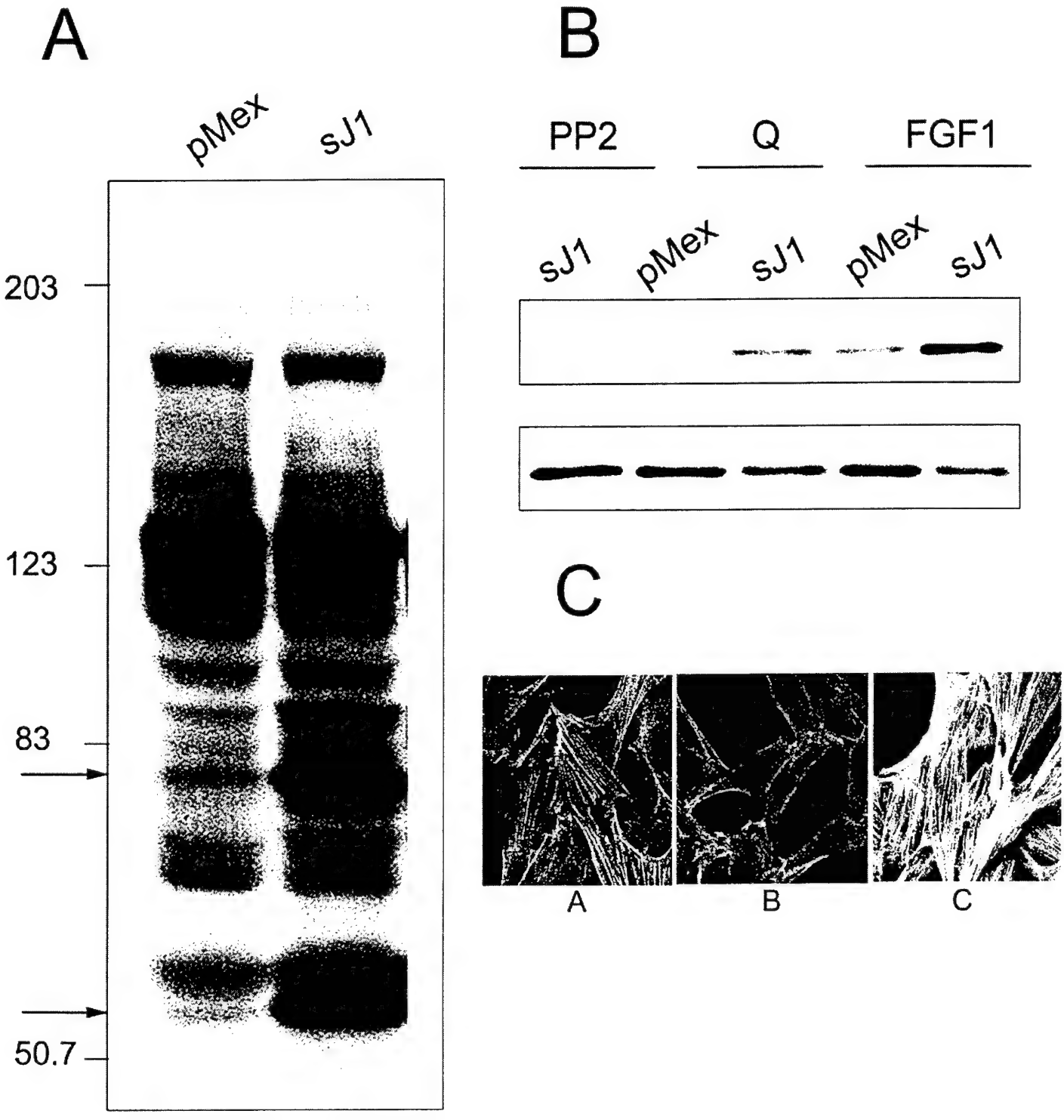
(C) were stained with CY3-phalloidin and analyzed by fluorescent confocal microscopy as described in the Experimental Procedures.

Figure 2. A. Cotransfection of dnSrc Abrogates Substrate-Dependent Chord Formation by sJ1 NIH 3T3 Cell Transfectants: Insert-less pMEXneo (A,D) and sJ1 (B,E) and dnSrc:sJ1 NIH 3T3 cell transfectants (C,F) were plated on either plastic (upper panel) or collagen Type 1 (lower panel) prepared as described in the Experimental Procedures and examined after 24 h incubation using an Olympus phase-contrast microscope (magnification 100x). **B. Cotransfection of dnSrc Restores the Expression of Pro-α-1 Collagen Type I in sJ1 NIH 3T3 Cell Transfectants:** The lysates of logarithmically growing pMEXneo, sJ1 and dnSrc:sJ1 NIH 3T3 cell transfectants were prepared and immunoblotted for pro-α-1 Collagen Type I as described in the Experimental Procedures. **C. Expression of dnSrc Decreases the Saturation Density of sJ1 NIH 3T3 Cell Transfectant Growth:** The growth dynamics of insert-less pMEXneo, sJ1 and dnSrc:sJ1 NIH 3T3 cell transfectants were determined as described under Experimental Procedures. Data are reported as viable cell number as a function of time.

Figure 3. A. Amlexanox Does Not Suppress the Collagen Type I-Dependent Chord Formation by sJ1 NIH 3T3 Cell Transfectants: Insert-less pMEXneo (A,C) and sJ1 (B,D) NIH 3T3 cell transfectants were plated in presence (C,D) or absence (A,B) of 0.3 mM amlexanox on Collagen Type I prepared as described under Experimental Procedures and examined after 24 h incubation using an Olympus phase-contrast microscope (magnification 100x). **B. The Expression of dnSrc in sJ1 NIH 3T3 Cell Transfectants Does Not Alter Their Impaired Migratory Activity.** Insert-less

pMEXneo, sJ1 and dnSrc:sJ1 NIH 3T3 cell transfectants were analyzed using the response to injury model of cell migration as described (24). Phase contrast photomicrographs (10x) were recorded at the times indicated.

Figure 4. Inhibition of MyoD-Mediated Transcription of a Luciferase Reporter Gene in sJ1 NIH 3T3 Cell Transfectants: Confluent monolayers of insert-less pMEXneo (M) and sJ1 NIH 3T3 cell transfectants were transiently transfected with 300 ng MyoD and 500 ng luciferase (Luc) reporter construct driven by the MCK promoter (MyoD). Some samples were also transfected with either 300 ng of N1IC, 500 ng of sJ1 or a combination of both N1IC and sJ1. Luciferase activity was measured with Promega's Dual-Luciferase Reporter Assay and the data are reported as fold-induction over background as determined by the activity of cells transfected with MCK-Luc reporter, only.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sJ1
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123
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PP2
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pMex
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A
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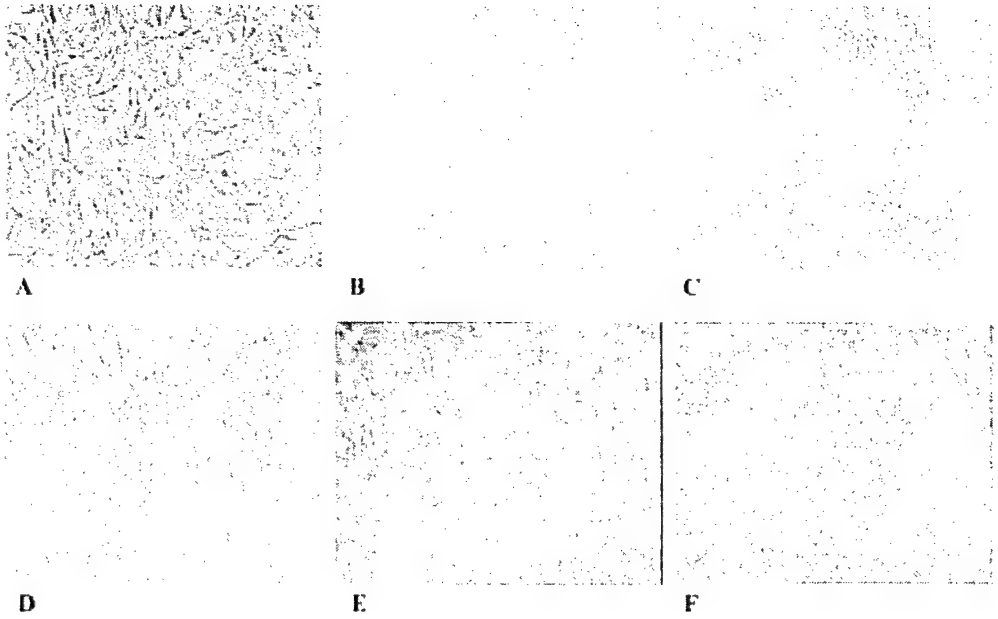
A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D
E
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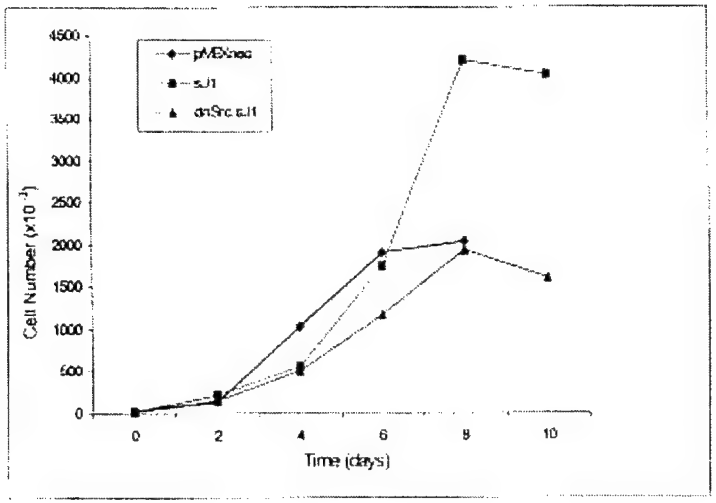
C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sJ1
dnSrc:sJ1
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Time (days)
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1500
2000
2500
3000
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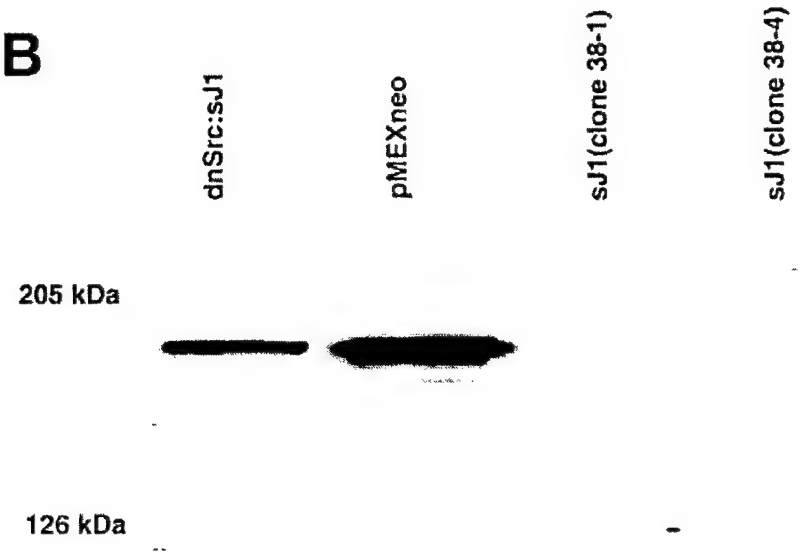
4000
4500
0
2
4
6
8
10

B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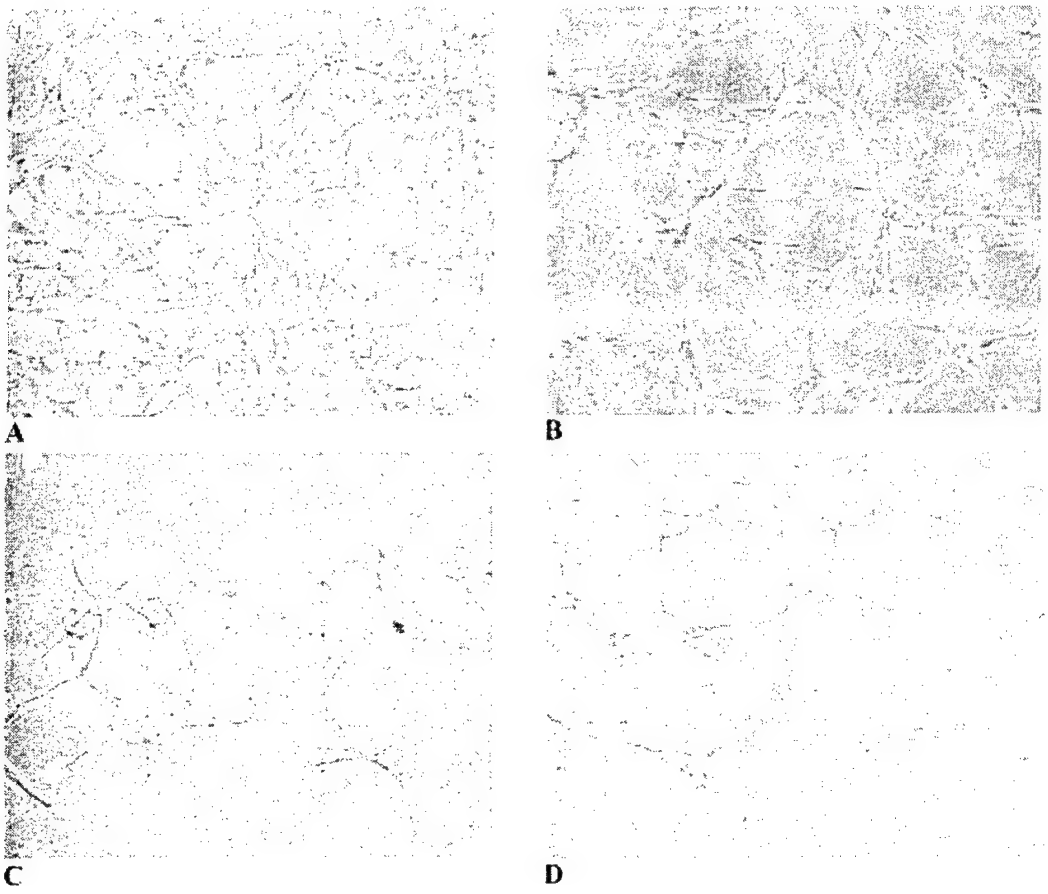
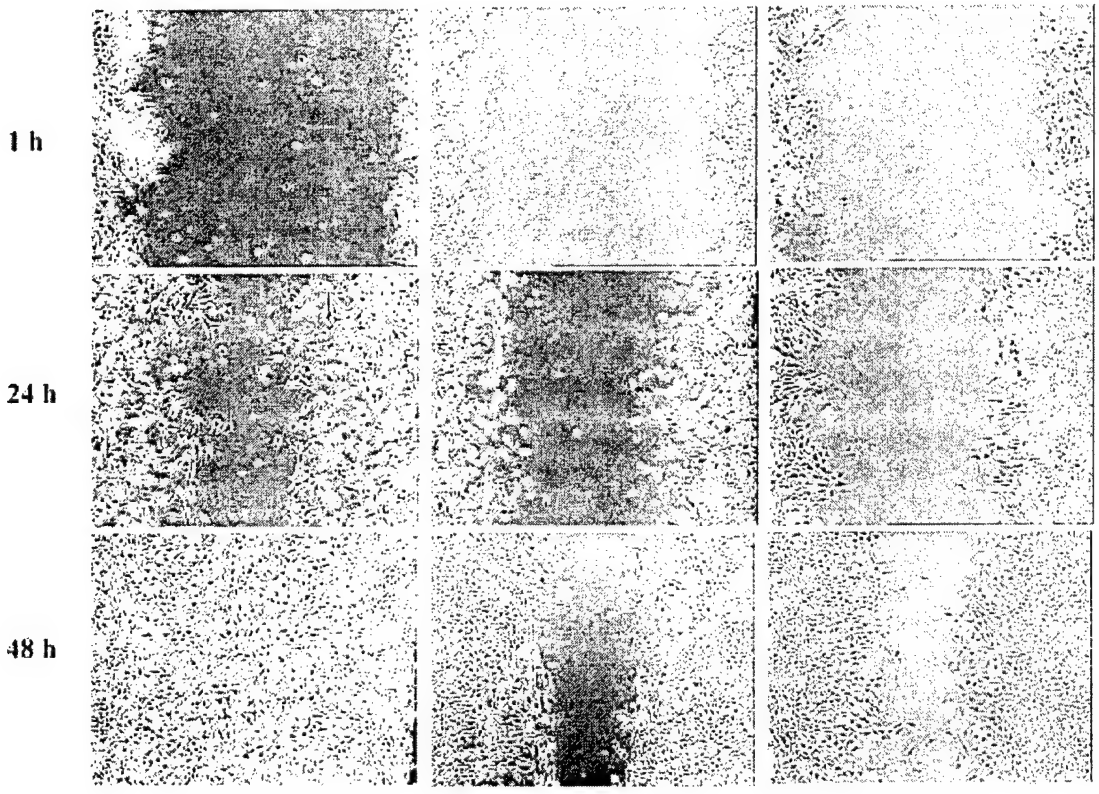
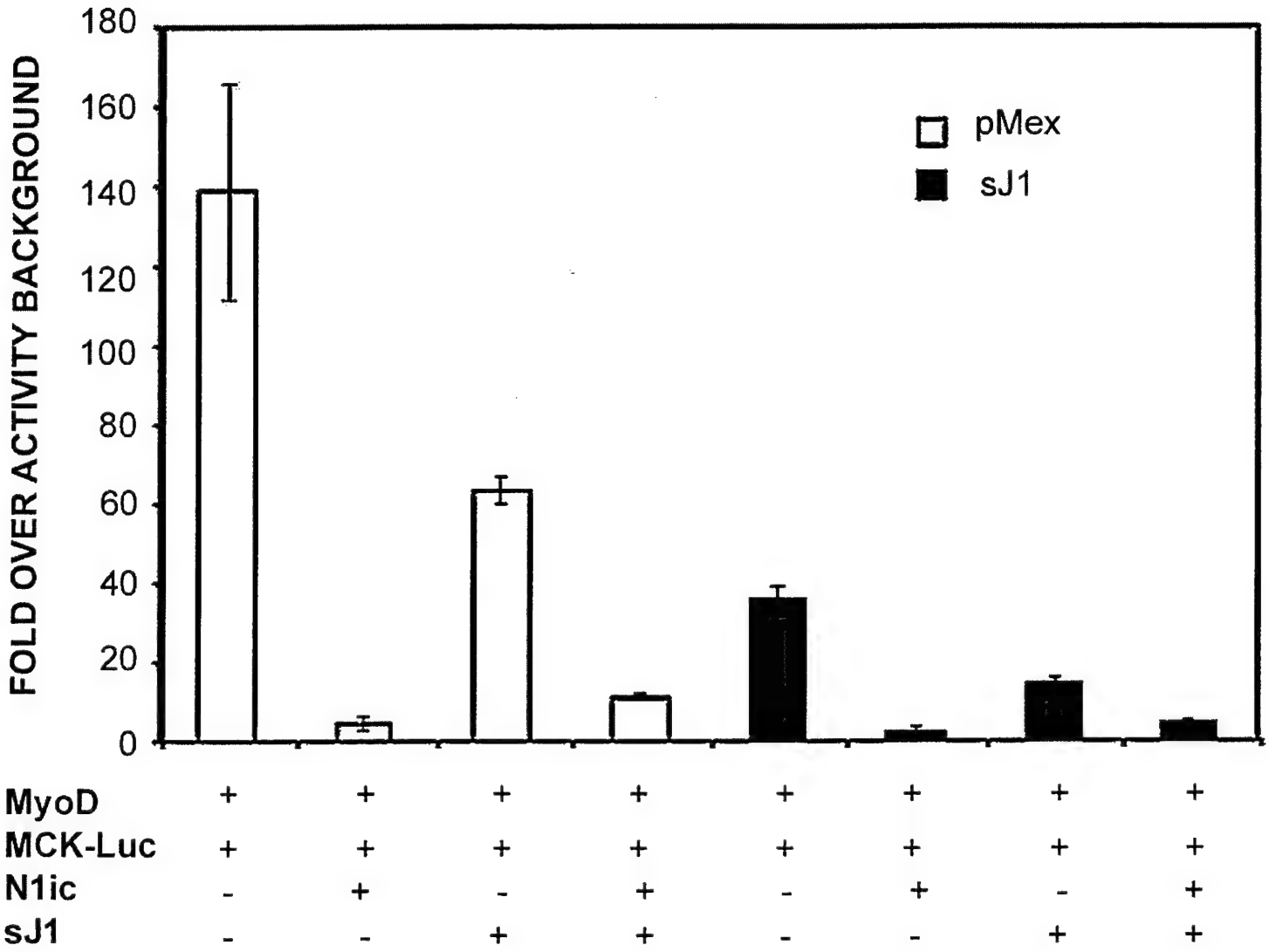
sJ1(clone 38-1)
sJ1(clone 38-4)
205 kDa
126 kDa

A



B




FOLD OVER ACTIVITY BACKGROUND
180
160
140
120
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80
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pMex
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sJ1 - - + + - - + +

To: Am. J. Pathol., 2001 (submitted 1/25/01)

Members of the *Jagged/Notch* gene families are expressed in injured arteries and regulate cell phenotype via alterations in cell-matrix and cell-cell interaction

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Running title: *Jagged/Notch* expression in injured vessels

28 pages, 1 table, 6 figures

This work was supported by AHA grant SDG9630017N (to L.L.), NIH grant RR15555 (to V.L., L.L., and T.M.), and NIH grant HL35627 (to T.M.).

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Abstract

The Jagged/Notch signaling pathways control cell fate determination and differentiation, and their dysfunction is associated with human pathologies involving cardiovascular abnormalities. To determine the presence of these genes during vascular response to injury, we analyzed expression of *Jagged1*, *Jagged2*, and *Notch1* through *4* following balloon catheter denudation of the rat carotid artery. Although low levels of *Jagged1*, *Jagged2*, and constitutive expression of *Notch1* were seen in uninjured endothelium, expression of all was significantly increased in injured vascular cells. High *Jagged1* expression was restricted to the regenerating endothelial wound edge, while *Notch* transcripts were abundant in endothelial and smooth muscle cells. To understand the basis for Jagged/Notch control of cellular phenotype, we studied an *in vitro* model of NIH3T3 cells transfected with a secreted form of the extracellular domain of Jagged1. We report that the soluble Jagged1 protein causes decreased cell-matrix adhesion and cell migration defects. Cadherin-mediated intercellular junctions as well as focal adhesions are modified in soluble Jagged1 transfectants, demonstrating that cell-cell contacts and adhesion plaques may be targets of Jagged/Notch activity. We suggest that Jagged regulation of cell-cell and cell-matrix interactions may contribute to the control of cell migration in situations of tissue remodeling *in vivo*.

Introduction

Notch receptor signaling is a conserved fundamental mechanism controlling cell fate during the development of many tissues, through interaction with ligands of the *Delta/Serrate* family (1,2). While extensive genetic studies have been performed in *Drosophila* and *C. elegans*, the mammalian paralogs have also been characterized to display similar complex functions. In humans, Notch1 through 4 comprise the receptor family, and Jagged1, Jagged2, and Delta1 are among the ligands. Interestingly, there have been at least three identified human disorders which are caused by altered function of components of the Jagged/Notch pathway. One of these leads to cell transformation and cancer, and the other two involve changes including defects in the cardiovasculature system. Chromosomal breakpoints in the *Notch1* gene have been shown to give rise to the overexpression of a truncated protein containing the intracellular portion of Notch1, leading to T cell acute lymphoblastic leukemias/lymphomas in patients (3,4). Mutations in the human *Jagged1* gene, in most cases leading to a truncated protein lacking transmembrane and cytosolic regions, cause the Alagille syndrome, a genetic disease characterized by liver failure, cardiac abnormalities and vertebral arch defects (5,6). Lastly, mutations in *Notch3* leading to point mutations in the extracellular domain of the Notch3 receptor have been found in patients with CADASIL (Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy), a condition characterized by recurrent subcortical strokes and progressive dementia (7,8). The identification of the genetic alterations involved in these

human diseases indicates that perturbation of Jagged/Notch signaling leads to dysfunctional cell and tissue behavior *in vivo*.

Murine genetic studies generating null mutations of the *Jagged/Notch* genes have indicated that the vascular system seems to be developmentally reliant on intact Notch signaling pathways. *Jagged1* null mutant mice display profound defects in the vasculature (9), and a *Notch1* null or processing deficient allele (10), as well as *Notch1/4* double mutants exhibit defects in vascular remodeling and angiogenesis (11). These observations in combination with the vascular defects seen in the human conditions where Notch3 signaling may be impaired suggest that responses to cardiovascular injury may also be regulated by *Jagged/Notch* gene family members.

We have previously characterized an *in vitro* system of stably transfected NIH3T3 cells expressing a soluble form of Jagged1 (12). The cDNAs for both the transmembrane form of Jagged1 as well as a variant lacking the transmembrane and intracellular regions were cloned from human umbilical vein endothelial cells undergoing *in vitro* angiogenesis (13), suggesting that cells may be able to produce variants of the Notch ligands. Previous studies have shown that this soluble form of Jagged1 promotes endothelial cell-like branching, inhibits the expression of collagen type I, abolishes contact inhibition of cell growth *in vitro*, and stimulates angiogenesis in a chick chorioallantoic membrane assay (12). We have further characterized the effects of the soluble Jagged1 protein with regards to characteristics

that are important in vascular cell remodeling, namely cell migration and interaction with neighboring cells and the underlying matrix, and report significant differences in cell behavior in the presence of the soluble Jagged1 protein.

Materials and Methods

In vivo tissue specimens

Vascular injury using balloon catheter denudation of rat carotid arteries was performed as described (14) with the approval of the Institutional Animal Care and Use Committee. *En face* specimens were prepared for *in situ* hybridization as previously described (15).

In situ hybridization

Full length cDNA clones for *Jagged1*, *Jagged2*, *Notch1* through *4* were a generous gift of G. Weinmaster (UCLA School of Medicine), and were used for the generation of ^{35}S-UTP labeled sense and antisense riboprobes. Tissue sections or *en face* preparations were treated with 20μg/ml proteinase K (Sigma Chem. Co., St. Louis, MO) before hybridization with 2 x 10^4 dpm/μl probe overnight at 50°C. Hybridized slides were treated with 20μg/ml Rnase A, then washed in a 50% formamide, 2x SSC, 20mM 2-mercaptoethanol buffer at 55-60°C.

Cell lines and tissue culture

The stable soluble Jagged1 NIH3T3 clones and the vector controls have been characterized previously (12), and were maintained in DMEM with 10% calf serum, 2mM L-glutamine, 50μg/ml gentamycin,

and 0.3mg/ml G418 at 37°C with 5% CO_2. For experiments, monolayers were removed with trypsin, and viable cells determined by the exclusion of trypan blue. Cells were counted and used for assays as described below.

Migration Assays

Cells were harvested by brief trypsin digestion and seeded at a density of 15,000 cells per cm^2 on a 6 well plate, allowed to grow to a confluent monolayer (24 hours) and then a scratch wound with a teflon comb (2.2 mm in diameter) was made the length of the dish as described (16). After the scratch, the wells were rinsed with PBS to remove detached cells then fed with growth medium. For studies of cell interaction with the matrix, plates were first coated with either PBS as a control or fibronectin (12) at 10μg/ml for 30 minutes prior to cell seeding. The peptide integrin inhibitor SM256 displays a high affinity and specificity for the αvβ3 integrin, although it can also inhibit GPIIb/IIIa, α5β1, and αvβ5 integrins at higher concentrations (17). In assays where inhibitor was used, SM256 was added to the assay with the cell suspensions at the given concentrations. Denuded area in μm^2 was evaluated using computer image analysis (NIH Image) at 24hr intervals until total closure of the denuded area was accomplished.

Immunostaining

For immunofluorescence staining, cells were plated on glass coverslips and fixed 24hr later with 4% paraformaldehyde in PBS. Fixed cells were blocked for 1hr in blocking buffer, BB (PBS, 0.1% Tween 20, 0.1% Triton X-100, 5% BSA), incubated for 1hr with primary antibodies (1μg/ml in BB), washed

with PBS, stained for 30min with secondary fluorochrome-conjugated antibodies (0.1μg/ml in BB) , washed with PBS, and embedded in 50% glycerol solution. We used monoclonal anti-vinculin antibodies (Sigma), monoclonal anti-phosphotyrosine antibodies (Upstate Biological Laboratories), monoclonal anti-β-catenin antibodies (Transduction Laboratories) and polyclonal anti-pan-cadherin antibodies (Sigma). As secondary antibodies we utilized anti-mouse IgG FITC- or CY3-conjugated goat antibodies (Sigma). Stained cells were studied and photographed using the fluorescent microscope (Zeiss) or the confocal fluorescent microscope, LTCS-SP (Leica).

Immunoprecipitation and immunoblotting

To study the activation of focal adhesion kinase (FAK), soluble Jagged1 transfectants and control vector-transfected cells were scraped in cold PBS containing 1mM sodium orthovanadate 2hr and 24hr after plating on plastic tissue culture dishes, and collected by centrifugation. Cell pellets were lysed in 0.5ml of cold lysis buffer (20mM Tris, pH 7.5, containing 300mM sucrose, 60mM KCl, 15mM NaCl, 5% glycerol, 2mM EDTA, 1% TritonX-100, 1mM PMSF, 2μg/ml aprotinin, 2μg/ml leupeptin, 0.2% deoxycholate, and 1mM sodium vanadate), and the lysates were clarified by centrifugation at 4°C. Lysates were rotated at 4°C for 1hr with 1 μg/ml rabbit anti-FAK antibodies (Sigma) followed by the addition of protein A Sepharose (Pharmacia Biotech) and further rotation for 1hr. The antibody complexes were washed three times with lysis buffer, and the immunoprecipitated FAK was eluted in 50μl SDS-PAGE sample buffer, resolved by 7.5% SDS-PAGE, transferred to Hybond C membrane (Amersham) and blotted with the monoclonal anti-phosphotyrosine monoclonal antibody (Upstate

Biotechnology). Phosphorylated FAK was visualized using a horseradish peroxidase-conjugated goat anti-mouse IgG antibody (BioRad) and the ECL system (Amersham). The FAK blots were stripped of the anti-phosphotyrosine antibodies using standard stripping buffer (18), reblotted with the anti-FAK antibodies, and FAK visualized using peroxidase-conjugated goat anti-rabbit IgG antibody (BioRad) and the ECL system (Amersham).

Results

Expression of Jagged/Notch gene family members following vascular injury in vivo

Since Jagged/Notch signaling has been implicated in control of blood vessel morphogenesis during embryogenesis (9,19) as well as during angiogenesis (12,13), we were interested in analyzing expression of these genes during large vessel repair. Utilizing the model of endothelial denudation in rat carotid arteries and aortae (20), we performed *in situ* hybridization to compare the expression of these genes in normal, uninjured endothelium compared to endothelial cells and smooth muscle cells responding to injury. Although the ligands *Jagged1* and *Jagged2* appeared to exhibit low levels of expression in normal endothelium, the expression of both genes was dramatically enhanced following endothelial denudation in the regenerating endothelial cells. This expression was seen predominantly in the migrating front of endothelial cells for *Jagged1*, and more diffusely for *Jagged2* (Fig. 1). The high levels of expression of both were maintained during the time period in which cells were actively migrating and proliferating, but were diminished at 4 weeks following injury, when cell proliferation

and migration have ceased (14,21,22). Likewise, we also observed that smooth muscle cells following vascular injury had increased expression levels of both *Jagged1* and *2*, in a time course similar to that of endothelial cells (Fig. 1 and Table I). However, compared to endothelial cells, smooth muscle cells exhibited much less *Jagged1* expression, while levels of the *Jagged2* transcript were high in injured smooth muscle cells (Table I). We also analyzed expression of the receptors *Notch1* through *4* in a comparable manner. In uninjured endothelium, *Notch1* was expressed constitutively, whereas *Notch2*, *Notch3*, and *Notch4* exhibited low levels of expression, strikingly similar to background (Fig. 2 and Table I). Although the levels of these Notch genes, particularly *Notch2* through *4* were increased in regenerating endothelial cells (Table I), endothelial expression was modest compared to the induction in injured intimal smooth muscle cells (Fig. 3). One interesting observation was that smooth muscle cell expression of both *Notch3* and *Notch4* appeared to be regulated by the presence of endothelial cells. While intimal smooth muscle cells in denuded areas expressed increased levels of the *Notch3* and *4* transcripts (Fig. 3, panels C, E), their expression was significantly upregulated in areas abutting the regenerating endothelial wound edge (Fig. 3, panels D, F). This suggests that interaction of endothelial cells with smooth muscle cells during vascular repair may contribute to the regulation of the levels of *Notch* receptor transcripts.

Soluble Jagged1 expression inhibits migration in NIH3T3 cells

Because remodeling vascular cells have the characteristic of being highly motile, we were interested in evaluating these features in cells overexpressing a soluble form of the Jagged1 protein. Using the

previously established *in vitro* model of NIH3T3 cells expressing a secreted form of the extracellular region of Jagged1 (12), we analyzed modifications in cell migration and phenotypic characteristics due to the expression of soluble *Jagged1*. Cells expressing the soluble *Jagged1* construct demonstrated a marked decrease in the rate of cell migration on plastic as compared to vector control transfectants (Fig. 4). The soluble Jagged1 transfectants appeared to maintain a highly defined wound edge with little invasion of individual cells into the denuded area (Fig. 4A). This effect was not seen with the vector controls, which rapidly demonstrated rogue infiltration to the denuded area followed by a quick (~24hr) disintegration of a defined wound edge. Soluble Jagged1 transfectants were able to migrate to close the denuded area, albeit at a much slower rate, approximately 24hr to 48hr following the repopulation of the vector control cells (Fig. 4B). Since the soluble Jagged1 transfectants were previously reported to display a spindle shape with decreased pseudopodia like processes (12), we evaluated the effects of different extracellular matrices on cell migration. When cells were seeded on a fibronectin substrate, cell spreading and attachment was restored, the soluble Jagged1 cells had a partial rescue of the migration defect, and individual cell migration into the denuded area was increased (Fig. 4C). The fibronectin substrate had no significant effect on the vector control transfectants (data not shown). We also inhibited cell interaction with the matrix using a peptide integrin inhibitor with selective, but not total specificity to the $\alpha v\beta 3$ integrin, SM256 (17), and observed that the peptide inhibited the migration of both the soluble Jagged1 transfectants as well as the vector controls, and their migration in the presence of the peptide was indistinguishable (Fig. 4D).

Regulation of cell-cell and cell-matrix interactions in soluble Jagged1 cells

Since our evaluation of cell migration included the observations that 1) the soluble Jagged1 cells maintained a greater degree of cell contact and migrated more as a sheet rather than the release of individual cells, and 2) the soluble Jagged1 cell migration defect could be minimized by increasing cell interaction with the matrix, we hypothesized that cell-cell and/or cell-matrix interactions were regulated by soluble Jagged1 production. We also observed that when plated during routine cell culture, the soluble Jagged1 transfectants had delayed cell spreading when compared to vector transfected controls (Fig. 5). Since activation and phosphorylation of the focal adhesion kinase occurs during attachment and spreading of cells *in vitro* (23), we used anti-phosphotyrosine blotting of immunoprecipitated focal adhesion kinase to evaluate this premise. Compared to vector transfected cells, the activation of focal adhesion kinase in soluble Jagged1 transfectants was delayed compared to control vector transfectants, with equal levels of phosphorylation only seen at later times after cell plating (Fig. 5).

As differences in the activation of the focal adhesion kinase might be related to distinct integrin levels in vector versus soluble Jagged1 transfectants, we performed a screen for cell surface levels of integrins, and found in general, similar levels of αv, $\alpha 5$, $\alpha v\beta 3$, $\alpha v\beta 5$, and $\alpha 5\beta 1$ on the surface of vector control and soluble Jagged1 transfected cells (data not shown). However, further analysis of the

focal adhesion complexes by immunofluorescence confirmed the biochemical differences in the soluble Jagged1 transfectants (Fig 6A-D). When cells were plated on plastic, vector-transfected control cells displayed abundant vinculin-positive focal adhesion sites. However, the soluble Jagged1 transfectants had significantly fewer focal adhesion sites and of smaller size. This difference was particularly exaggerated when the cells were plated on a collagen substrate (Fig. 6C, D), a condition previously shown to support the endothelial cell-like branching morphology of the soluble Jagged1 transfectants (12). Immunostaining with antibodies against phosphotyrosine, a well known histochemical marker of focal adhesion sites (23) yielded results similar to those described for vinculin staining (data not shown). The defects in the focal adhesion sites are consistent with the delayed cell spreading and slower phosphorylation in the soluble Jagged1 transfectants described above. The attenuation of focal adhesion sites may at least partially explain the decreased migratory activity of the soluble Jagged1 transfectants. However, the decrease in motility could be also attributed to the strengthening of intercellular contacts, and the highly defined wound edge in the soluble Jagged1 transfectants supports this explanation. To explore the effect of soluble Jagged1 transfection upon cell-cell contacts, we performed immunofluorescence staining of confluent cultures of vector and soluble Jagged1 transfectants using a pan-cadherin antibody which detects all cadherins, or an antibody against β-catenin. The immunofluorescence preparations were studied using confocal microscopy under standard conditions of illumination and registration, which permitted the objective comparison of the cell clones. We found a significant increase of both cadherins and β-catenin expression in the cell-cell

contacts of soluble Jagged1 expressing cells compared to vector controls (Fig. 6E-H). These findings provide evidence for the regulation of both cell-matrix and cell-cell adhesion molecules by endogenous Notch signaling through soluble Jagged1.

Discussion

Although members of the *Jagged/Notch* gene families have been well documented to be expressed during embryonic development in several vertebrate species (24-28), there has been less described regarding normal expression and especially during tissue repair. We were particularly interested in vascular repair since the human diseases which have been associated with mutations in *Jagged/Notch* genes frequently involve cardiovascular abnormalities (29). As such, we hypothesized that the endogenous expression of these genes may play a role in these processes, and that a perturbation of the balance of signals may lead to human pathologies. Our observations are the first to show that both smooth muscle cells and endothelial cells of the vasculature greatly increase the expression of these genes *in vivo* following injury, and that levels of *Notch* receptor expression may be related to endothelial cell/smooth muscle cell interaction. In general, while *Notch1* and *Jagged1* and *2* were found expressed at low levels in normal endothelium, there was no expression of *Notch2* through *4*. All genes were induced following injury, and it is interesting to note that the expression of *Notch* receptors appeared to be higher in smooth muscle cells in regions of contact with endothelial cells. While it has been observed that the phenotype of intimal smooth muscle cells *in vivo* appears regulated by the

presence of regenerating endothelial cells (30,31), the molecular basis for this interaction has not been established. We propose that cell-cell interactions between Jagged ligands in endothelial cells and Notch receptors in intimal smooth muscle cells may be one mechanism of regulating smooth muscle cells at the denuded endothelial cell border.

Our *in vitro* studies have focused on a system utilizing a secreted form of the extracellular portion of the Jagged1 ligand in NIH3T3 cells in an effort to understand how perturbation of the Notch signaling system affects cell phenotype. Recent data have suggested that the active form of a Delta/Serrate/Jagged ligand may be more highly regulated than previously expected. In addition to the transmembrane bound ligand, Delta has been shown to be cleaved from the cell surface, generating a soluble agonist for Notch activity (32). Conversely, secreted forms of Delta and Serrate have been shown to act as dominant negative forms of the ligands in *Drosophila* embryos (33), and immobilization of the extracellular domain of Delta was shown to be required for Notch-mediated inhibition of myoblast differentiation and HES1 transactivation (34). The initial rationale for producing the secreted form of the Jagged1 ligand was the discovery of this form expressed by human endothelial cells undergoing *in vitro* angiogenesis (13). Indeed, previous studies in this system have verified that the production of the soluble Jagged1 form changes cell morphology, decreases contact inhibition of cell growth, and stimulates angiogenesis in a chick chorioallantoic membrane (12). As soluble Delta ligand has been shown to be generated *in vivo*, proteolytic cleavage of the Jagged1 ligand may also be

a mechanism for the regulation of function, and it will be critical to establish the active forms of these ligands during normal embryonic development as well as disease processes.

Our findings that the presence of the soluble Jagged1 protein decreases cell adhesion and migration, probably as a result of inhibiting the formation or stability of focal adhesion complexes, have implications for understanding downstream events involved in vascular repair. In addition, the increases in β-catenin and cadherins in the intercellular junctions of soluble Jagged1 transfectants provides a consistent explanation for the decrease rates of migration in the soluble Jagged1 population. Increased expression of cadherin has been implicated in contact mediated inhibition of cell migration (35) as well as acting as a tumor suppressor for growth and invasion of tumors *in vitro* and *in vivo* (36-38). Interestingly, cellular migration and invasion are key features of remodeling vascular cells. The observations that soluble Jagged1 protein inhibits cell matrix interaction, focal adhesion formation, and cellular migration while increasing cell-cell contacts suggests that endogenous Jagged/Notch signaling may act to maintain cell interaction with the matrix and to activate the migratory ability of cells, possibly by decreasing cell-cell contacts. This interpretation is consistent with the *in vivo* expression of *Jagged/Notch* genes particularly at the leading wound edge in regenerating endothelium, where cells are actively migrating to cover the denuded surface. Also particularly in the smooth muscle cell population, one would expect that invasion of cells through the internal elastic lamina would require the attenuation of cell-cell contacts and an increase in cell-matrix interaction to allow singly migrating

cells to enter the intimal compartment. Although cadherins and focal adhesions have not been established as direct downstream targets of Notch signaling, recent data have shown that 1) perturbation of Notch signaling in *Xenopus* embryos leads to changes in the segmental expression pattern of the paraxial protocadherin (PAPC), which is expressed during convergence extension cell movements in gastrulating embryos (39,40), and 2) expression of a constitutively activated Notch4 receptor disrupts contact inhibition of proliferation in mammary epithelial cells *in vitro*, and stimulates invasion and migration into a collagen gel (41). These studies are consistent with our observations that both cell-matrix and cell-cell interactions can be influenced by the Jagged/Notch pathway. Our results in combination with the earlier report of soluble Jagged1 cells regulating angiogenesis in the chick chorioallantoic membrane assay (12) demonstrate that both microvessel and large vessel phenotype are controlled through Notch signaling. We would predict that in large vessels *in vivo*, the expression of the *Jagged/Notch* genes reflect a functional role in modulating these processes in cellular migration and invasion.

Acknowledgements

The authors would like to thank G. Weinmaster (UCLA) and S. Mousa (DuPont) for generously providing reagents. We also thank C.H. Tenney and M. Sullivan for their work on the *in vitro* cell assays.

Gene	Normal endothelium	8d injured endothelium	8d injured SMC
Jagged1	+	++++ wound edge	+
Jagged2	+	+++	++
Notch1	++	++	+
Notch2	+/-	++	+++
Notch3	+/-	++	++
Notch4	+/-	++	++

Table I: Summary of expression of *Jagged/Notch* genes in the vessel wall *in vivo* following injury. At 8d after injury, the levels of proliferation in both endothelial cells and smooth muscle cells are at a peak, and both cell types are actively migrating. *In situ* hybridization was performed with antisense riboprobes to the *Jagged/Notch* genes, and expression qualitatively determined as compared to sense riboprobe controls.

Figure Legends

Figure 1. Expression of *Jagged* in normal and injured vessels *in vivo*. *In situ* hybridization with the sense (A) or antisense probes (B-H) for Jagged1 (A-D) and Jagged2 (E-H) was performed on *en face* preparations of vessels as indicated. Although normal expression for both genes in uninjured vessels was low (B, E), transcripts were upregulated in both injured endothelium (EC, C, F) and smooth muscle cells (SMC, D, H). Transcripts were again reduced to background levels in a stable lesion (G). Original magnification 400x.

Figure 2. *Notch* expression in uninjured endothelial cells *in vivo*. *In situ* hybridization was performed on *en face* preparations of normal endothelium using antisense probes for *Notch1* through *4*. Constitutive but low expression for *Notch1* transcript was detected, whereas background levels of *Notch2-4* were seen (B-D). Original magnification 200x.

Figure 3. Expression of *Notch* in injured SMC *in vivo*. *In situ* hybridization was performed using antisense probes for *Notch1-4* on *en face* preparations of vessels 8d after injury. Depending on the area of the vessel examined, SMC were either alone (A-C, E) or adjacent to the endothelial wound edge (D, F). All *Notch* genes were found to be abundantly expressed in injured SMC at 8d as well as 2wks following injury. Original magnification 200x.

Figure 4. Production of soluble Jagged1 inhibits NIH3T3 cell migration. Stable transfectants of vector control or soluble Jagged1 expressing clones were assayed for migratory ability using a scrape assay and measuring the migration of cells onto the denuded surface as described (16). A) Photomicrographs show representative fields of the clones at 24hr and 48hr following the scrape injury. Original magnification 100x. B) Quantitation of the denuded area reflected a 24-48hr lag in the repopulation of the denuded area in soluble Jagged1 transfectants compared to vector controls. C) When the assay was performed on fibronectin (FN) coated plates (10μg/ml), the migration defect of the soluble Jagged1 cells was partially rescued, with migration intermediate between migration of vector control and soluble Jagged1 cells plated on plastic. D) Inclusion of 100mM SM256, a peptide integrin inhibitor, into the assay caused a reduction in the migration of both vector control and soluble Jagged1 cells.

Figure 5. Delayed cell spreading and FAK phosphorylation in soluble Jagged1 cells. A) Cells were plated at equal cell densities on plastic and photographed after 1hr, 3hr, and 5hr. Note delayed cell spreading in soluble Jagged1 cells. Original magnification 200x. B) Cell lysates were collected as described from vector control or soluble Jagged1 transfectants at 2hr and 24hr after plating on plastic. Lysates were immunoprecipitated using anti-FAK antibodies and subjected to SDS-PAGE and Western blot analysis with an anti-phosphotyrosine antibody (top panel). Blots were stripped and re-probed with anti-FAK antibodies (lower panel).

Figure 6. Alterations in focal adhesions and cell-cell adhesions in soluble Jagged1 cells. Immunofluorescent staining was performed for the proteins indicated on vector control (left column) and soluble Jagged1 (right column) transfectants. Focal adhesions were demonstrated by anti-vinculin staining of cells on plastic (A-B) or plated on collagen (C-D). Arrowheads show focal adhesion plaques. Cell-cell contacts were visualized using a pan-cadherin antibody (E-F) and an anti-β-catenin antibody (G-H). Original magnification 1000x.

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Fig. 1

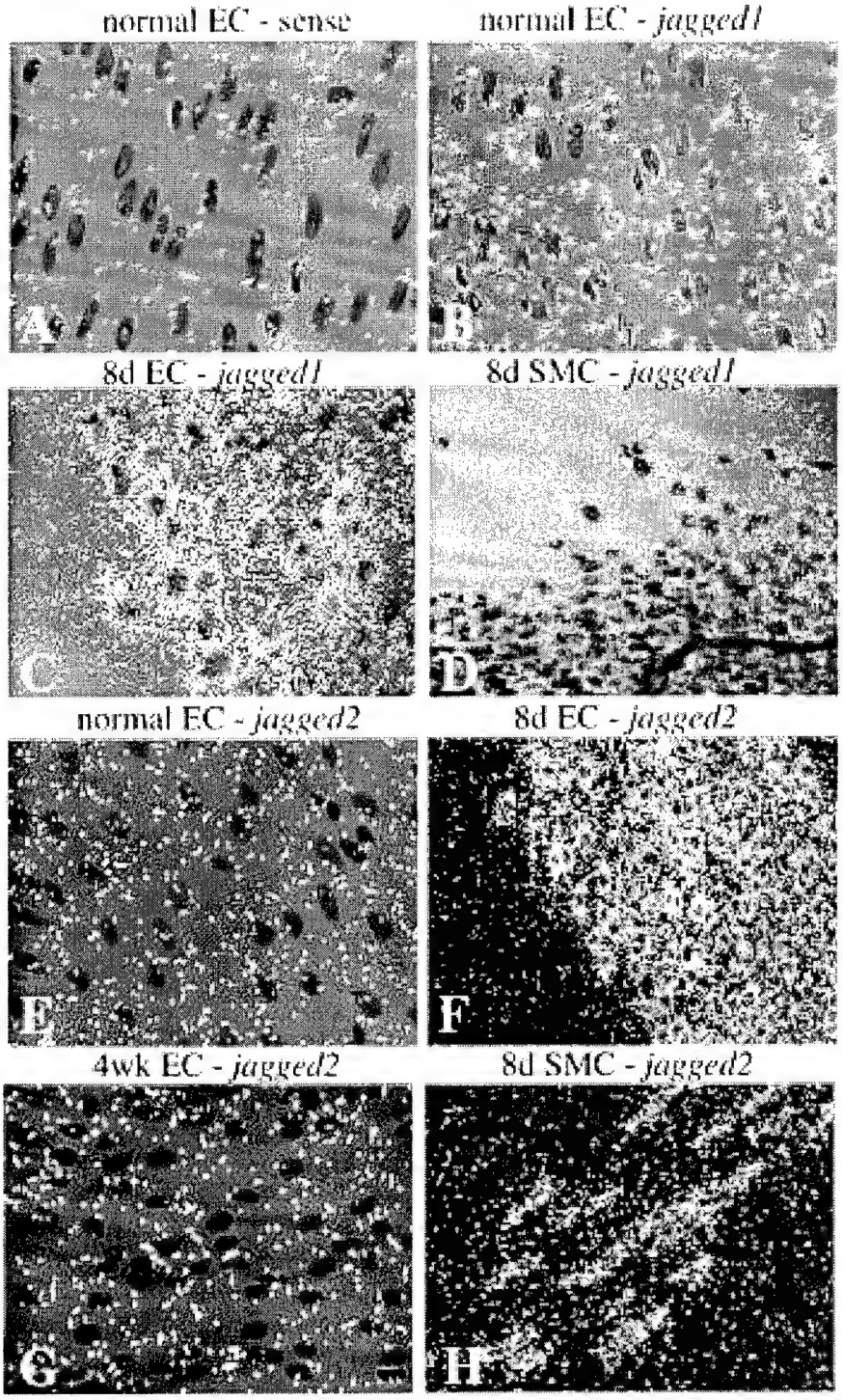

normal EC - sense
normal EC - jagged1
A
B
8d EC - jagged1
8d SMC - jagged1
C
D
normal EC - jagged2
8d EC - jagged2
E
F
4wk EC - jagged2
8d SMC - jagged2
G
H

Fig. 2

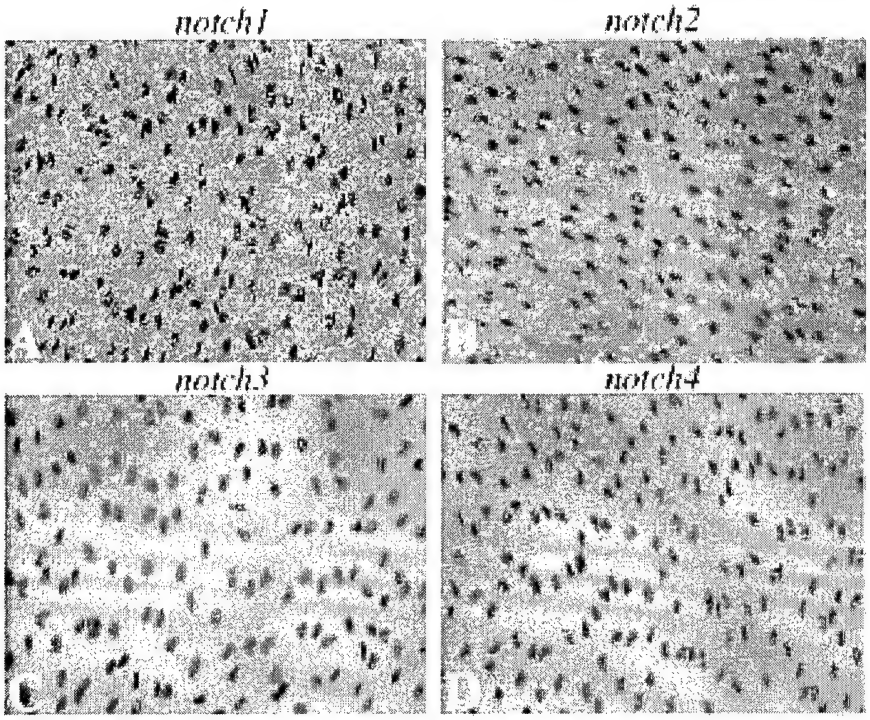


Fig. 3

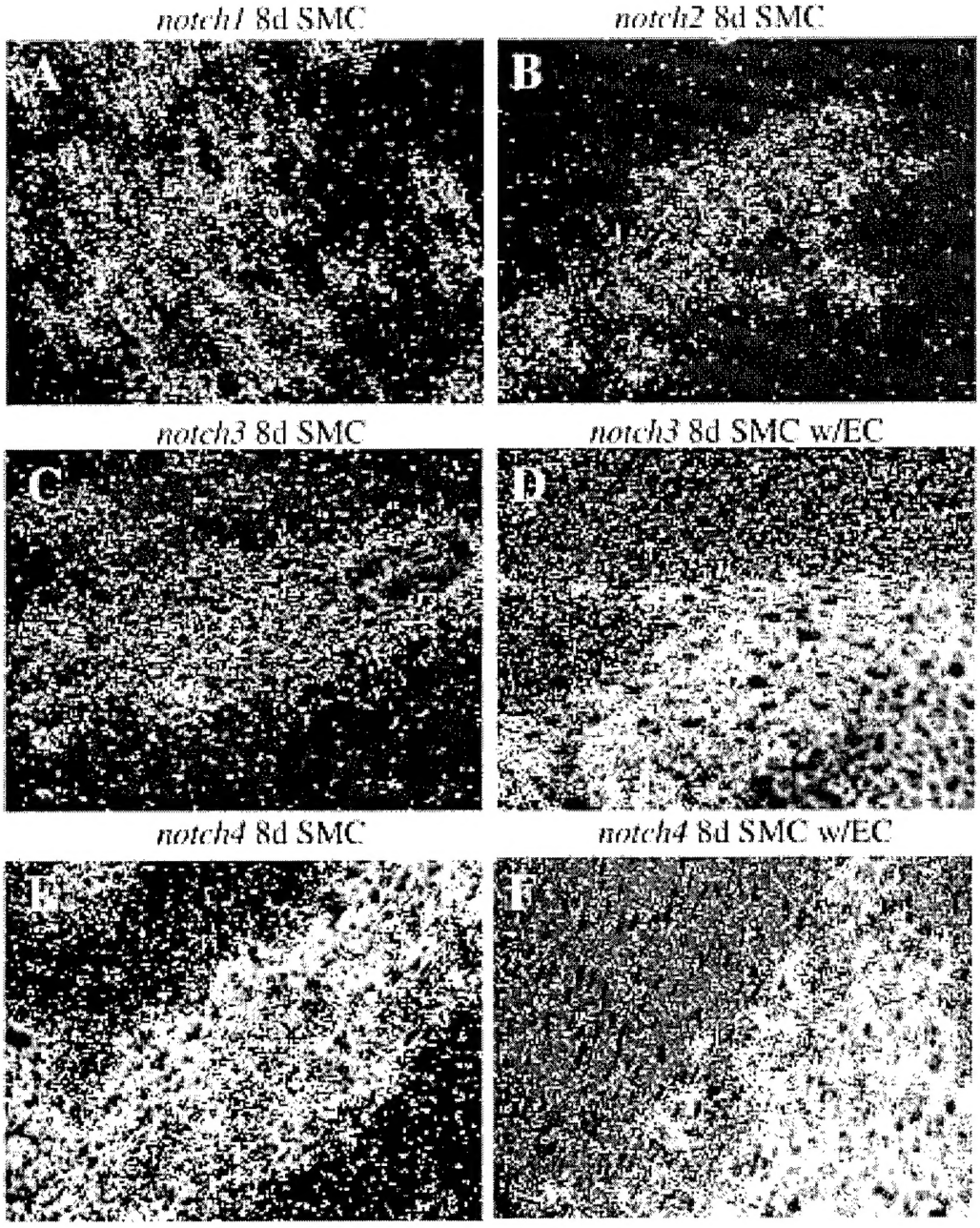

notch1 8d SMC
A
notch2 8d SMC
B
notch3 8d SMC
C
notch3 8d SMC w/EC
D
notch4 8d SMC
E
notch4 8d SMC w/EC
F

Fig. 4

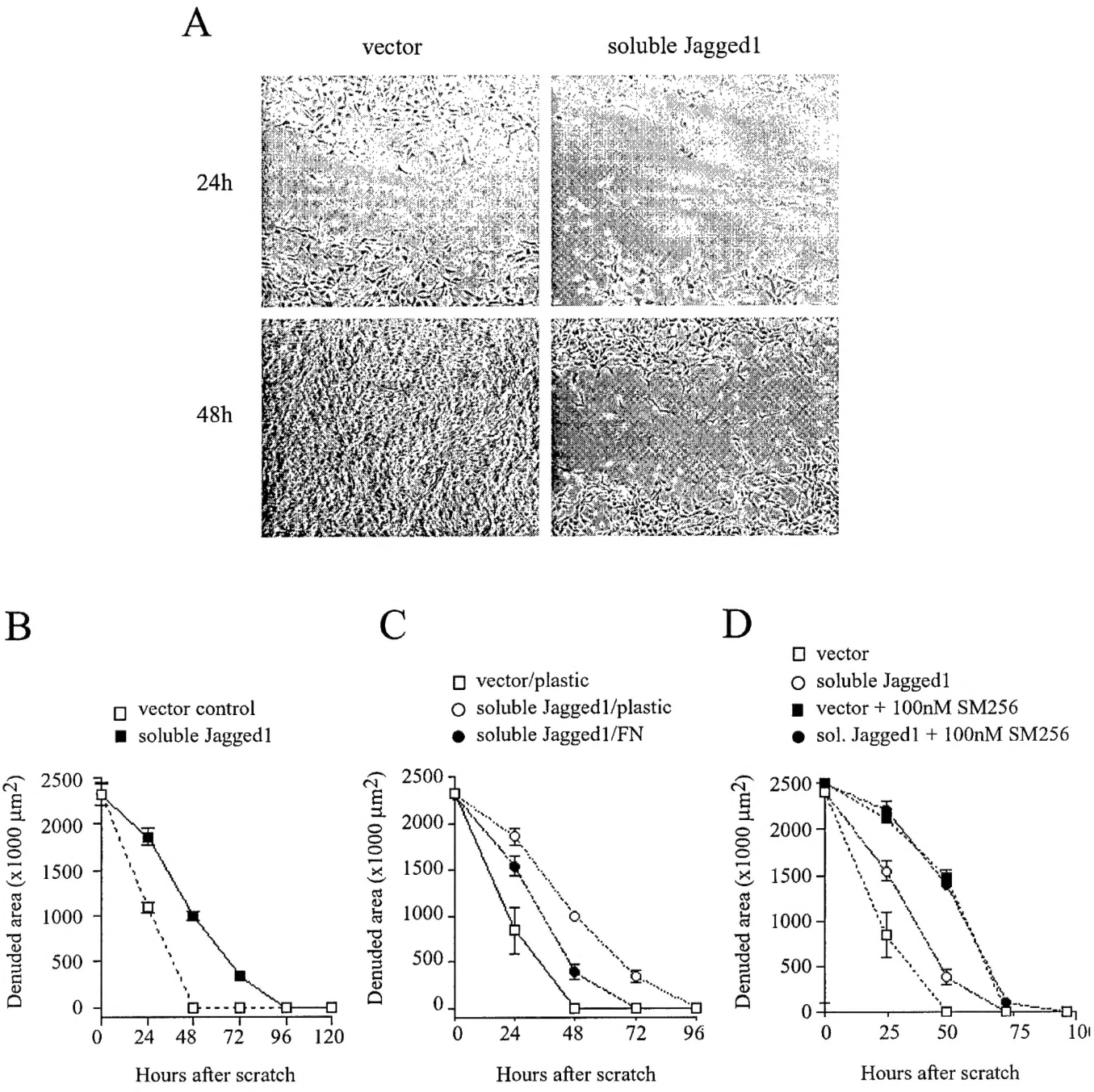
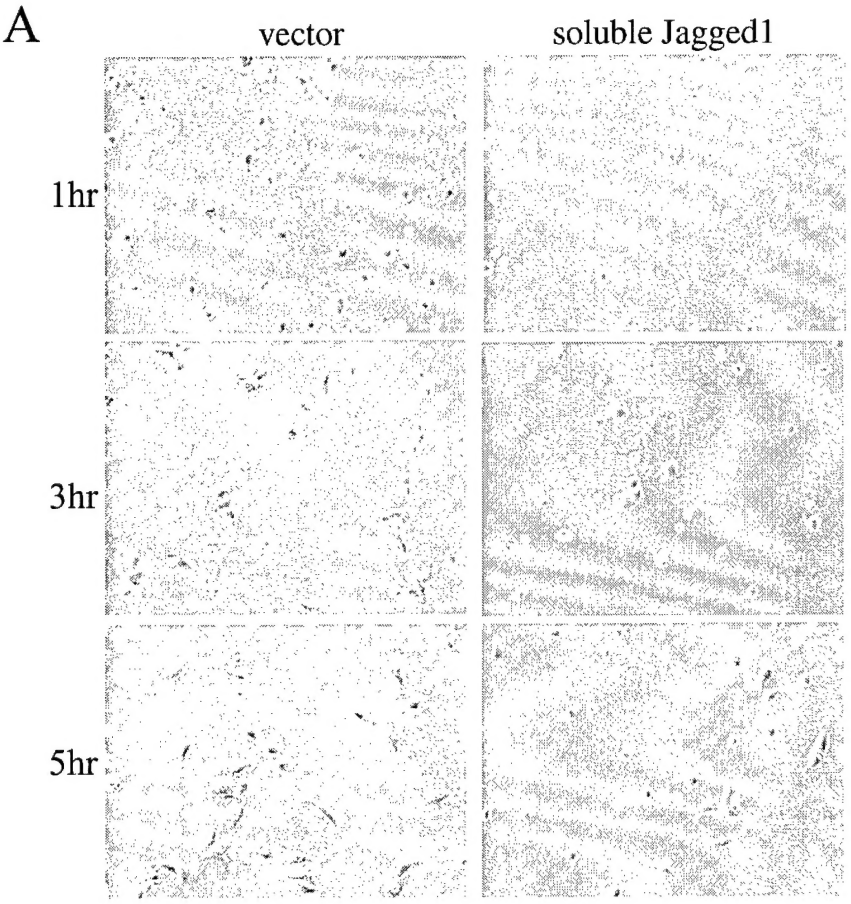

A
vector
soluble Jagged1
24h
48h
B
vector control
soluble Jagged1
Denuded area (x1000 µm2)
2500
2000
1500
1000
500
0
0
24
48
72
96
120
Hours after scratch
C
vector/plastic
soluble Jagged1/plastic
soluble Jagged1/FN
Denuded area (x1000 µm2)
2500
2000
1500
1000
500
0
0
24
48
72
96
Hours after scratch
D
vector
soluble Jagged1
vector + 100nM SM256
sol. Jagged1 + 100nM SM256
Denuded area (x1000 µm2)
2500
2000
1500
1000
500
0
0
25
50
75
Hours after scratch

Fig. 5


A
vector
soluble Jagged1
1hr
3hr
5hr

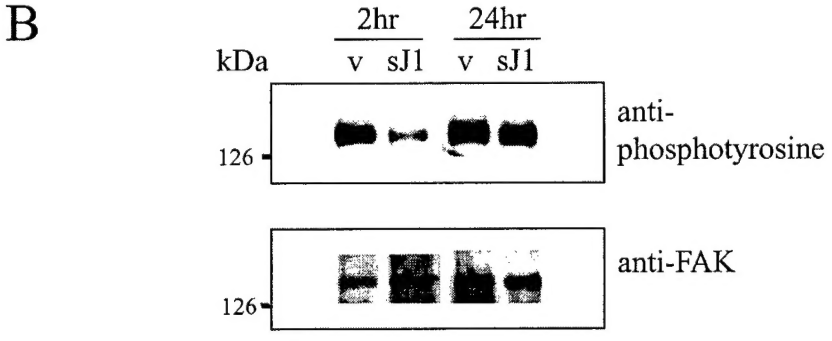
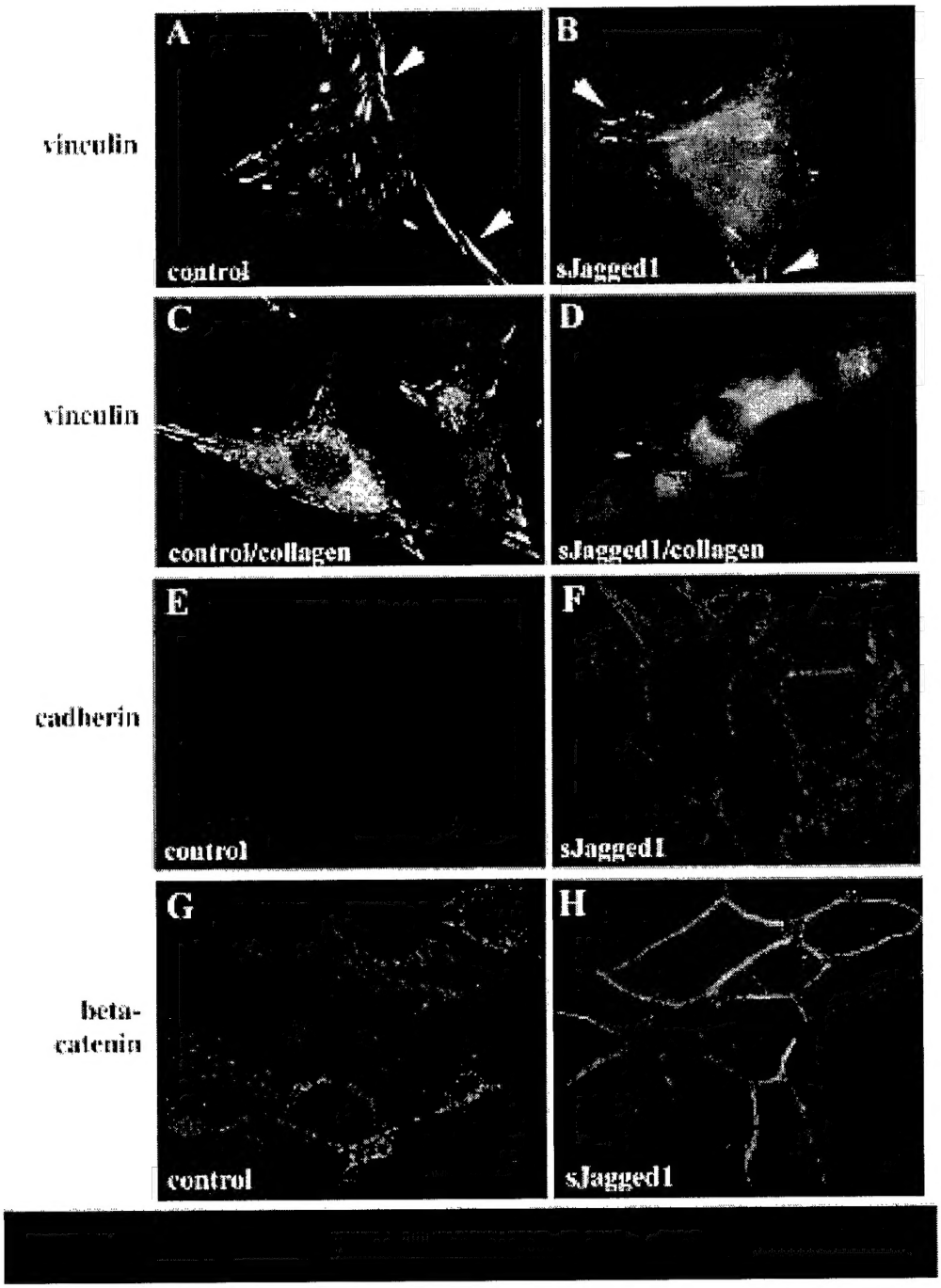

B
2hr
24hr
kDa
v
sJ1
v
sJ1
126
anti-
phosphotyrosine
126
anti-FAK

Fig. 6


A
B
vinculin
control
sJagged1
C
D
vinculin
control/collagen
sJagged1/collagen
E
F
cadherin
control
sJagged1
G
H
beta-
catenin
control
sJagged1